Academic Year 2007-2008

Laboratory for Parasitology

Department of Virology, Parasitology and Immunology

Ghent University – Faculty of Veterinary Medicine

Study of *Ostertagia ostertagi* excretory-secretory products

Heidi Saverwyns

Proefschrift voorgelegd aan de faculteit Diergeneeskunde tot het behalen van de graad van Doctor in de Diergeneeskundige Wetenschappen

Promotoren: Prof. Dr. E. Claerebout en Dr. P. Geldhof

Dank u wel

Het heeft wel eventjes geduurd, maar nu is het zover. Veel mensen hebben de afgelopen jaren direct of indirect hun medewerking verleend aan het tot stand komen van dit proefschrift. Een aantal van hen wil ik hier persoonlijk bedanken.

In eerste instantie wens ik Prof dr. Jozef Vercuysse en mijn promotor Prof. Dr. Edwin Claerebout te bedanken omdat jullie mij de kans boden om op het labo Parasitologie te doctoreren. Jullie deur stond altijd open zowel in goede tijden als in slechte tijden. Bedankt voor alles!!

Peter, nog maar pas terug uit Moredun en vervolgens gebombardeerd tot mijn promotor. Jouw hulp was onmisbaar bij het vervolledigen van dit proefschrift. Hopelijk heb je niet te veel fietsuren moeten missen door de ontelbare corrigeersessies. Denk vooral aan die 'oh zo leuke bureau momenten' wanneer we samen neurieden op mijn nieuwste ringtone.

Dr. Marc Fransen, bedankt voor alle hulp en goede tips wanneer mijn fagen niet deden wat van hen verwacht werd. Natuurlijk wil ik u ook bedanken voor het gedetailleerd en supersnel nalezen van dit proefschrift!

Isabel, jouw hersenspinsels (neergepend in een FWO aanvraag) betekenden het startsignaal van een wilde rit op het faagdisplay rad. Je hebt ons reeds enkele jaren geleden verlaten, desalniettemin was je direct bereid om in mijn leescommissie te zetelen. Bedankt hiervoor!

Vervolgens zou ik ook alle leden van de examen commissie willen bedanken voor het kritisch nalezen van mijn proefschrift en de constructieve opmerkingen.

Mijn mede-parasitologen, samen hebben we gezorgd voor de vrolijke noot op het labo, soms zo luid dat we zelfs geen radio nodig hadden. Ok, ik beken, maar Bruno, jij neemt ongetwijfeld de job van koorleider met evenveel enthousiasme over. Yves, Prisca, Abdel, Johannes, Thomas, Lonneke, Pierre ... ook jullie hebben mij 'overleefd' dus het enigste wat mij nu nog rest is enkelen veel succes toe te wensen met hun doctoraat terwijl ik de rest van jullie een fantastische carrière toewens in binnen- en buitenland! Aline en Iris, heel erg bedankt voor al het werk (gedurende de laatste maanden). Door jullie lijken RNAi en ELISA een makkie. Annelies, we zijn tezamen begonnen en gaan nu ook samen eindigen dit jaar, veel succes met jouw doctoraat! Stijn, Nathalie en Rudy, die shitty bussiness, ik mis het nu al (jullie waarschijnlijk minder). Parasitologen, jullie zijn altijd welkom voor een schantwis/sandwich in

Evergem maar neem jullie woordenboek mee en PS. der is een omleiding langs Kortrijk, hé.

Mieke, de administratieve steunpilaar van de para maar voor mij was je nog veel meer dan dat: mijn persoonlijke celeb gossip magazine, een huisapotheek, een ISPC compagnon, een tante kaat met gouden raad…. Kortom onmisbaar.

Dirk, onze redder in nood als de domme PCs het niet deden, ook jij verdient een schouderklopje. We hebben ons zo geamuseerd en deze laatste weken heb je me geholpen met alle drukwerk. Merci!!

Den immuno staat garant voor veel lachen en een gratis uitleendienst van centrifuges dus dat is zeker een mercike waard. Kristien, veel succes met je nieuwe job en tot fuifs. Annelies, veel succes met jouw doctoraat. Griet, veel succes met de nieuwe bébé (zal nu wel al geboren zijn).

Annie, bedankt voor de babbels en het opblinken van onze bureau zoals nooit tevoren.

Veerle, mijn oude bureaugenoot. Nu is het mijn beurt om te doctoreren en hierin was ook jouw hulp onmisbaar (vooral niet te vergeten: het toffe thesisjaar). We hebben ons toch geamuseerd in ons prachtig hoekkantoortje.

Ma en Pa, bedankt voor alles. Ik weet niet waar te beginnen. Om te beginnen, hebben jullie mij de kans gegeven om te verder te studeren maar jullie hebben ook steeds gezorgd voor oppeppende woorden en de opvang van de kindjes. Rudy en Dorine, ook jullie wil ik bedanken voor het immer paraat staan voor ons gezin en bij ziekte van Maite en Rune. Ze konden altijd terecht bij hun Mémé, Oma en 2 Pépé's. Het heeft de combinatie gezin/docteren veel makkelijker gemaakt. Wendy, Marc en Barbara, ook jullie hebben je steentje bijgedragen. Bedankt hiervoor!

Aan al mijn vrienden, een knieval om jullie te bedanken voor de randanimatie en let op, nu mijn doctoraat af is, I'm back to party.

Maite en Rune, mijn twee schetebezekes. Nu beseffen jullie het nog niet maar ik wil hier toch een kleine boodschap achterlaten. Jullie komst heeft mijn leven serieus op zijn kop gezet, in de positieve zin weliswaar. Jullie glimlach hielp me elk moeilijk moment te overwinnen. Dikke duim van jullie mama.

Yves, mijn ventje, ik heb het te weinig gezegd maar zonder jou was dit doctoraat niet mogelijk geweest! Bedankt voor jouw onvoorwaardelijke steun gedurende de voorbije 10 jaar en laat de rest maar komen...

Heidi

List of Figures

List of tables

List of abbreviations

AA	Amino acid
Actb	β-actin
AEG	Acidic epididymal glycoprotein-like molecule
AL	ASP-like protein
ANS	8-Anilino-1-naphthalenesulfonic acid
ASP	Activation-associated secreted protein
ATP	Adenosine Triphosphate
BL	Blank
Bp	Base pair
cDNA	Complementary deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
Ds	Double stranded
DTT	Dithiothreitol
ECL	Enterochromaffin-like
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EPG	Eggs per gram
ES	Excretory-secretory
ESP	Excretory-secretory product
EST	Expressed sequence tag
ES-Thiol	Thiol-binding adult excretory-secretory antigens
EX	Extract
F	Adult female worms
FEC	Faecal egg counts
G-17	Gastrin-17
G-34	Gastrin-34
Gapdh	Glyceraldehyde-3-phosphate dehydrogenase
GI	Gastrointestinal
GNAS	Guanine nucleotide-binding protein Gs {alpha} subunit
HBSS	Hanks' balanced salt solution
HS	Horse serum
I	Intestine
Ig	Immunoglobulin
IL	Interleukin
IVR	*In vitro* released
L	Larva
L3+	Third stage larva with sheath
L3-	Third stage larva without sheath
LBAT	Luria Bertani medium with ampicilline and tetracycline
M	Adult male worms
MAPPIT	Mammalian Protein-Protein Interaction Trap
MHC	Major histocompatibility complex
ML	Macrocyclic lactones
mRNA	Messenger ribonucleic acid
MS	Mass spectrometric
MW	Molecular weight
N	Native
Nlp	Neuropeptide-like protein
NPA	Nematode polyprotein allergen
NPY	Neuropeptide-Y
OD	Optical density
OPA	*Ostertagia* polyprotein allergen
ORF	Open reading frame
P4H	Prolyl-4-hydroxyprolylase
PBS	Phosphate buffered saline

PBST	Phosphate buffered saline with Tween 20
PCR	Polymerase chain reaction
PDI	Protein disulphide isomerase
PDB	Protein Data Bank
PGE	Parasitic gastroenteritis
Ph	Pharynx
PI	Isoelectric point
PR1	Pathogen related protein
PVDF	Polyvinyldine difluoride
Q-PCR	quantative polymerase chain reaction (real-time PCR)
RACE	Rapid amplification of cDNA ends
RNA	Ribonucleic acid
RNAi	RNA interference
Rpm	Rotations per minutes
RT-PCR	Reverse transcriptase polymerase chain reaction
scFv	Single chain antibody fragment
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
Sf	Sphincter
sHSP	Small heat shock protein
ssDNA	Single stranded desoxyribonucleic acid
T(3)	Triiodothyronine [^{125}I]-T_3
T(4)	Thyroxine [^{125}I]-T_4
Th2	T-helper 2 cell
TNF	Tumor necrosis factor
Tpx	Testis specific proteins
TRP	Transthyretin-related protein
TTL	Transthyretin-like protein
TTP	Triacylglycerol transfer protein
TTR	Transthyretin
Tubb	β-tubulin
YT	Yeast Extract Tryptone

General introduction to *Ostertagia ostertagi*

1. Introduction

Infections with gastrointestinal nematodes influence livestock world wide and result in parasitic gastroenteritis (PGE). This disease represents a substantial economic burden on the cattle industry due to production losses. Clinical PGE mainly affects calves during their first grazing season and is characterised by watery diarrhoea, weight loss, a dull hair coat, anorexia, a general loss of condition and eventually death (Anderson *et al*., 1965; Hilderson *et al*., 1987). Subclinical infections mainly occur in older cattle where they are associated with reduced milk production and carcass weight (Entrocasso *et al*., 1986; Gross *et al.,* 1999).

In the following paragraphs, a short overview will be given of the life cycle, epidemiology and pathology of the most important gastrointestinal nematode in cattle in temperate climate regions, *Ostertagia ostertagi*, as well as on the possible control systems used today against this parasite.

1.1. Life cycle and epidemiology of *Ostertagia ostertagi*

O. ostertagi is a member of the *Trichostrongyloidea* (ordo *Strongylida*) and has a direct life cycle which consists of two stages: the free-living stage on pasture (pre-parasitic) and the parasitic stage in cattle. Eggs from mature females in the abomasum are passed in the faeces. These hatch in the faecal pat to first stage larvae (L1). The L1 larvae grow and moult to second stage larvae (L2). Subsequently the L2 larvae moult to become infective third stage larvae (L3). The L3s retain the cuticle from the second stage (L2) as a protective sheath, but can survive for long periods within the faecal pat. The time taken to develop into infective larvae is dependent on favourable stimuli such as temperature and humidity (optimal conditions: 2 weeks). The parasitic stage of the *Ostertagia* life cycle in cattle begins when L3 larvae are ingested during grazing. In the rumen the L3 larvae lose their protective sheath and pass on to the abomasum where they penetrate the gastric glands. After exsheathment to L4 and subsequently to L5 larvae, the young adults can emerge from the gastric glands and continue their maturation on the mucosal surface. The normal prepatent period is 21 days. However, under certain circumstances, ingested L3 larvae can stop their maturation as inhibited L4 larvae (hypobiosis) and this may last for as long as 6 months. Clearly, when arrested development occurs the prepatent period will be prolonged (Fig. 1).

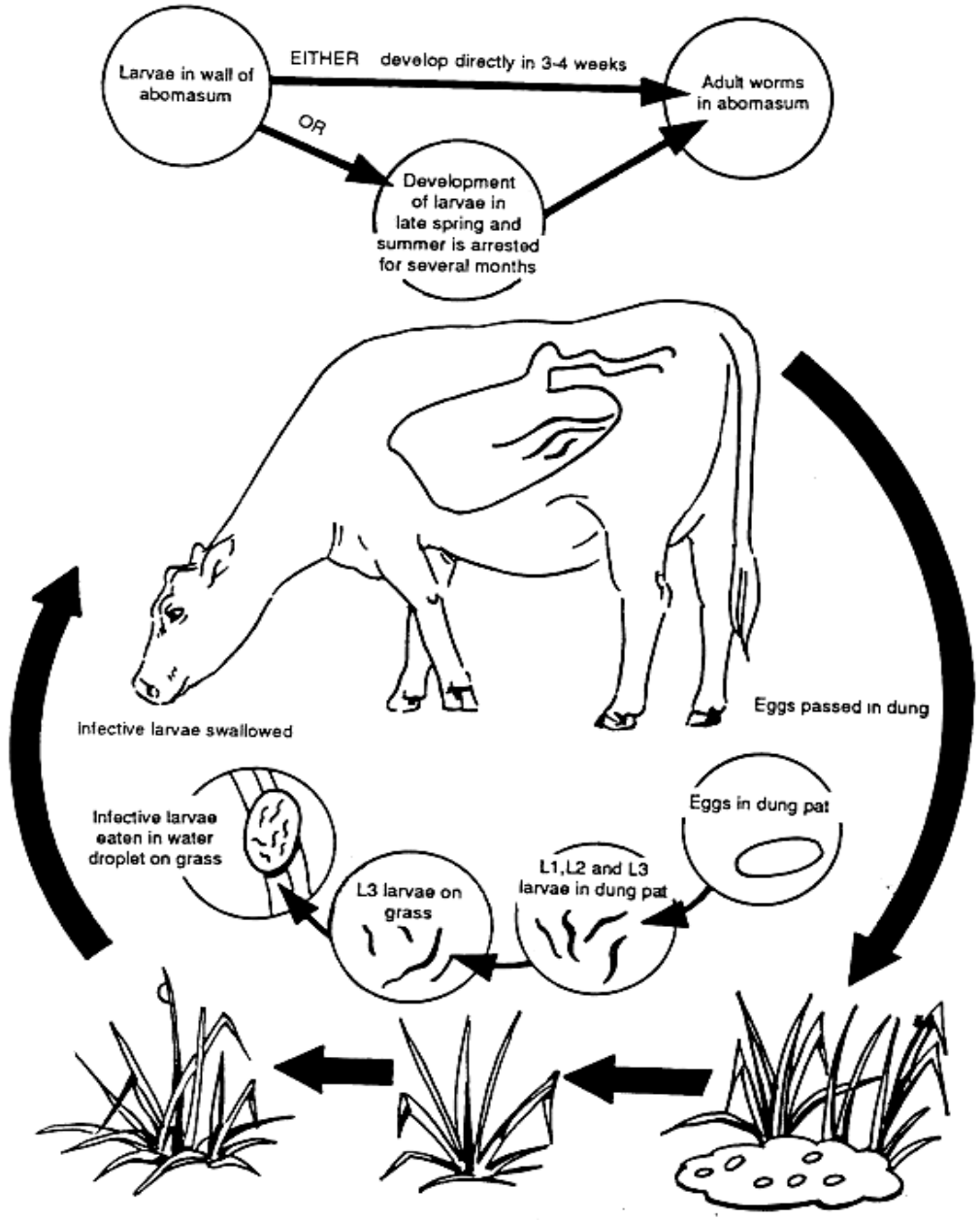


Figure 1: Life cycle of *Ostertagia ostertagi*: pre-parasitic and parasitic stage, adapted from Petalia ™ & © 2000 Petsite.com Ltd; http://www.petalia.com.au

Nematode infections in spring are derived largely from over-wintered larvae on pasture but in some cases may consist of a few over-wintered adults or worms maturing from inhibited larvae. Infective larvae are ingested by animals that are turned out in the beginning of the new grazing season (May/June) (Fig. 2). After three weeks, eggs are shed and they develop to L3 larvae. At this time of year, the hatching of eggs is rather slow but it becomes more rapid towards mid-summer as the temperature rises. The majority of the eggs deposited during April, May and June will reach the infective stage from mid July onwards. This is called the 'mid-summer rise'. The exact period in which this occurs is variable and depends on the weather (Hilderson *et al*., 1987). When the summer is very

dry, larvae will accumulate in faecal pats and there will be little or no release. Wet weather conditions in autumn will provoke the massive release of these larvae and will lead to a very high pasture contamination. When temperatures fall and autumn progresses, an increasing proportion of ingested L3 larvae will only develop to the L4 stage and then go into larval arrested development (or hypobiosis). In late autumn calves can therefore harbour thousands of these "early L4" larvae but few developing forms or adults.

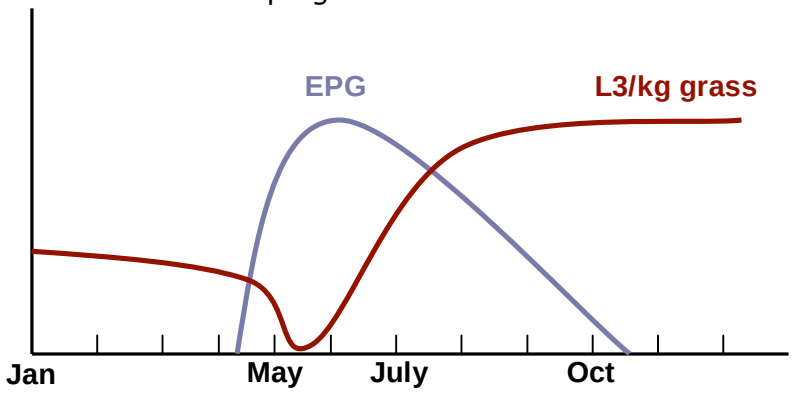


Figure 2: A diagram showing the egg output per gram faeces (EPG) and pasture contamination with L3 larvae (L3/kg grass).

1.2. Clinical and economic importance

Clinical ostertagiosis normally occurs in first grazing season calves and older animals that are infected for the first time. Subclinical infections mainly occur in adult cattle. The primary clinical sign in bovine ostertagiosis is watery diarrhoea and is usually accompanied by reduced appetite, anorexia, dehydration and thirst. Infected animals are characterised by dull, rough coats and hindquarters soiled with faeces as a result of the profuse diarrhoea (Anderson *et al*., 1965; Hilderson *et al*., 1987). Weight loss may be significant.

Clinical ostertagiosis can be observed under two sets of circumstances referred to as type I and type II diseases.

Type I disease occurs in young cattle grazing on pastures for their first time during the period of high pasture contamination (July-October). The pathological and clinical signs are due to the direct development of large numbers of L3 larvae to adult worms over a relatively short period of time (approximately 3 weeks).

Type II disease in yearlings and older cattle always results from arrested L4's resuming their development to immature adults (March-May) and leaving the gastric glands weeks or months after being ingested as L3s.

About 88 million cattle (Eurostat, 2006) in the EU are exposed to gastro-intestinal nematodes during grazing. Infections with these nematodes place a substantial burden on the livestock economy. *Ostertagia* mainly affects yearlings

and results in a slower growth pattern and costly anthelmintic treatments. In older cattle, ostertagiosis manifests itself as a sub-clinical disease. However, it has been demonstrated that subclinical GI-nematode infections can have a negative impact on milk yield (Gross *et al*., 1999; Sanchez *et al.*, 2004). Carcass quality is also influenced by trichostrongyle infections, with reduced carcass weight, killing out percentage and related carcase measurements (Entrocasso *et al*., 1986). Finally, the repeated anthelmintic treatments represent also a big cost for the farmers.

1.3. Control

Control of parasitism in Europe is mainly focussed on the use of anthelmintics (Vercruysse and Dorny, 1999). They are strategically used at therapeutic doses at those times when parasites abundantly contaminate the pasture. Macrocyclic lactones (ML), broad spectrum anthelmintics, have obtained their leading position in treatment against *Ostertagia* based on their high efficacy and persistent activity (Vercruysse and Rew, 2002). The availability of new generic macrocyclic lactones (ivermectin) coupled to the zero withdrawal time for milk resulted in the increased use of ML. However, this intensive and preventive use of ML over the past two decades entails several drawbacks e.g. high costs of repeated anthelmintic treatments, negative effects on the development of natural immunity against gastrointestinal nematodes (Vercruysse *et al.*, 1994; Vercruysse and Claerebout, 1997) but also the consumer awareness of possible chemical residues in food and environment (Wall and Strong, 1987; Strong, 1993). More importantly, the increasing incidence of anthelmintic resistance poses a serious threat on the future routine use of anthelmintics (Vermunt *et al*., 1995; Coles *et al*., 1998; Fiel *et al*., 2001). At the present time there has been one report on anthelmintic resistance in *Ostertagia* in cattle in New Zealand (Waghorn et al., 2006). However it is not unlikely that ML resistance could develop further in the *O. ostertagi* population. This assumption has stimulated the search for alternative control measures.

Vercruysse and Dorny (1999) proposed several non-chemotherapeutic control options: exploiting breed resistance to parasite infection, changes in pasture management, nematophagous fungi, dietary supplementation with protein and grazing of plants containing anthelmintic substances such as condensed tannins (Waller, 2006) and finally vaccination. However, none of these options have yet reached the stage of commercialisation in cattle due to the lower efficacies and more variable results in the control of nematode infections than anthelmintics. Moreover, these alternative control measures are currently only being evaluated in first-season grazing calves and their effects in adult cattle remain undetermined (Jackson and Miller, 2006).

2. References

Anderson, N., Armour, J., Jarrett, W.F., Ritchie, J.S. and Urquhart, G.M. (1965). A field study of parasitic gastritis in cattle. Vet Rec 77, 1196-1204.

Coles, G.C., Stafford, K.A. and MacKay, P.H. (1998). Ivermectin-resistant *Cooperia* species from calves on a farm in Somerset. Vet Rec 142, 255-256.

Enterocasso, C.M. Parkins, J.J., Bairden, K. and McWilliam, P.N. (1986). Production, parasitological and carcase evaluation studies in steers exposed to trichostrongyle infection and treated with a morantel bolus or fenbendazole in two consecutive grazing seasons. Res Vet Sci 40, 76-85

Eurostat (2006). http://epp.eurostat.ec.europa.eu/

Fiel, C.A., Saumell, C.A., Steffan, P.E. and Rodrigues, E.M. (2001). Resistance of *Cooperia* to ivermectin treatments in grazing cattle of the Humid Pampa, Argentina. Vet Parasitol 97, 211-217.

Gross, S.J., Ryan, W.G. and Ploeger, H.W. (1999). Anthelmintic treatment of adult dairy cows and the effect on milk production. Vet Rec 144,581-587

Hilderson, H., Berghen, P., Dorny, P. and Vercruysse, J. (1987). Bovine ostertagiosis; part I: A review. Vl diergen tijdschr 98, 195-214.

Jackson, F. and Miller, J. (2006). Alternative approaches to control--quo vadit? Vet Parasitol 139, 371-84.

Sanchez, J., Markham, F., Dohoo, I., Sheppard, J., Keefe, G. and Leslie, K. (2004). Milk antibodies against *Ostertagia ostertagi*: relationships with milk IgG and production parameters in lactating dairy cattle. Vet Parasitol 120, 319-30.

Strong, L. (1993). Overview – The impact of avermectins on pastureland ecology. Vet Parasitol 48, 3-17.

Vercruysse, J., Hilderson, H. and Claerebout, E. (1994). Effect of chemoprophylaxis on immunity to gastrointestinal nematodes in cattle. Parasitol Today 10, 129-32.

Vercruysse, J. and Claerebout, E. (1997). Immunity development against *Ostertagia ostertagi* and other gastrointestinal nematodes in cattle. Vet Parasitol 72, 309-16

Vercruysse, J. and Dorny, P. (1999). Integrated control of nematode infections in cattle: A reality? A need? A future? Int J Parasitol 29, 165-175.

Vercruysse, J. and Rew, R.S. (2002). Macrocyclic lactones in antiparasitic therapy. (eds. J. Vercruysse and R.S. Rew) @ CAB *International* 2002.

Vermunt, J.J., West, D.M. and Pomray, W.E. (1995). Multiple resistance to ivermectin and oxfendazole in *Cooperia* species of cattle in New Zealand. Vet Rec 137, 43-45.

Waghorn, T.S., Leathwick, D.M., Rhodes, A.P., Jackson, R., Pomroy, W.E., West, D.M. and Moffat, J.R. (2006). Prevalence of anthelmintic resistance on 62 beef cattle farms in the North Island of New Zealand. N Z Vet J 54, 278-82.

Wall, R. and Strong, L (1987). Environmental consequences of treating cattle with the antiparasitic drug ivermectin. Nature 327(6121):418-21.

Waller, P.J. (2006). From discovery to development: current industry perspectives for the development of novel methods of helminth control in live stock. Vet Parasitol 139, 1-14.

Chapter 1 Literature review:

Pathogenesis of ostertagiosis in relation to excretory-secretory products

1. Introduction

During the development of gastrointestinal nematodes from L3 to adult worms several important morphological, physiological and biochemical changes are observed in the abomasal mucosa of ruminants (Murray *et al*., 1970; McKellar, 1993; Fox, 1997). Infections with gastrointestinal (GI) nematodes induce strong Th2-like mucosal immune responses characterized by accumulation of local antibodies, mast cells and eosinophils. However, despite these quick changes the protective immunity against GI nematodes like *Haemonchus* and *Cooperia* develops slowly and requires prolonged periods of exposure. In case of *O. ostertagi*, even after years of exposure the immunity is not sterile (Gasbarre, 1997; Gasbarre *et al*., 2001).

It has been postulated that excretory-secretory products (ESPs) of the nematode might play an important role in modulating the abomasal environment and the host immune response (Simpson *et al*., 1997, 1999; Scott *et al*., 2000). Numerous *in vitro* and *in vivo* studies have assessed the role of parasite ESPs in the pathogenesis. Effects on gastrin and pepsinogen secretions, pH, gastrointestinal motility and immune responses have been reported for GI nematodes such as *Teladorsagia circumcincta* (Scott and McKellar, 1998c; Simpson *et al.*, 1999; Lawton *et al*., 2002; Wildblood *et al*., 2005) and *Haemonchus contortus* (Merkelbach *et al*., 2002; Huber *et al*., 2005; Wildblood *et al*., 2005). However, data on the involvement of *O. ostertagi* ESPs in the pathogenesis are scarce and mainly focussed on their immunomodulatory properties (Washburn and Klesius, 1984; Klesius *et al*., 1986; De Marez *et al*., 2000; Gomez-Munoz *et al*., 2004). Furthermore, the underlying mechanisms of the host-parasite interaction are largely unknown. Identification of ESPs and their host ligands remains a challenging task.

Therefore, this chapter will address two major points. The first part will deal with different aspects of the pathogenesis of ostertagiosis. The second part will focus on the relationship between *O. ostertagi* ESPs and the pathogenesis and further characterization of known *O. ostertagi* ESPs that might have a crucial role in the host-parasite homeostasis.

2. Pathogenesis of ostertagiosis

The pathogenic effects of *Ostertagia* are due to the growth of developing larvae in gastric glands and the emergence of immature adults from these glands. Initially, penetration of the infective L3 larvae in the abomasal mucosa causes erosion of the gland cells (Murray *et al*., 1970). This results in a reduced number of functional gastric glands in the *Ostertagia*-infected abomasum. The parasitized gland is swollen, giving it a nodular appearance at post mortem inspection (Figure 1.1). The folds of the abomasum may be oedematous and reddened. This loss of function results in a number of effects: a substantional

rise in pH of abomasal contents, a reduced bacteriostatic effect, elevated plasma pepsinogen levels, hypoalbuminaemia and hypergastrinaemia (McKellar, 1993; Figure 1.2). This will be discussed in detail in the following paragraphs.

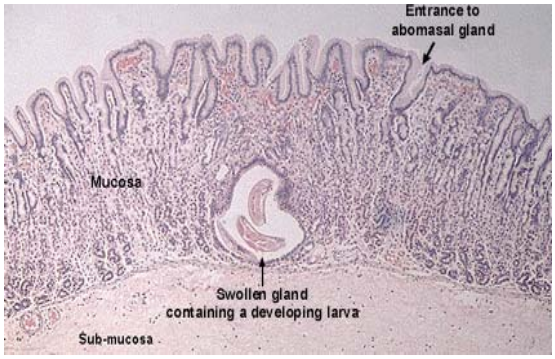
A.  **B.** 

Figure 1.1 A. Nodular appearance of parasitized glands **B.** Transverse tissue section of developing *Ostertagia* L4 larvae in the gastric glands (7-10 days after infection), from 'Parasites and Parasitic Diseases of Domestic Animals' by Dr. Colin Johnstone © 1998 University of Pennsylvania.

The net clinical effect of all these changes is that large amounts of osmotically active substances - bacteria, undigested proteins and plasma proteins (especially albumin) - congregate in the abomasum and these will promote the passage of water into the gut producing (watery) diarrhoea.

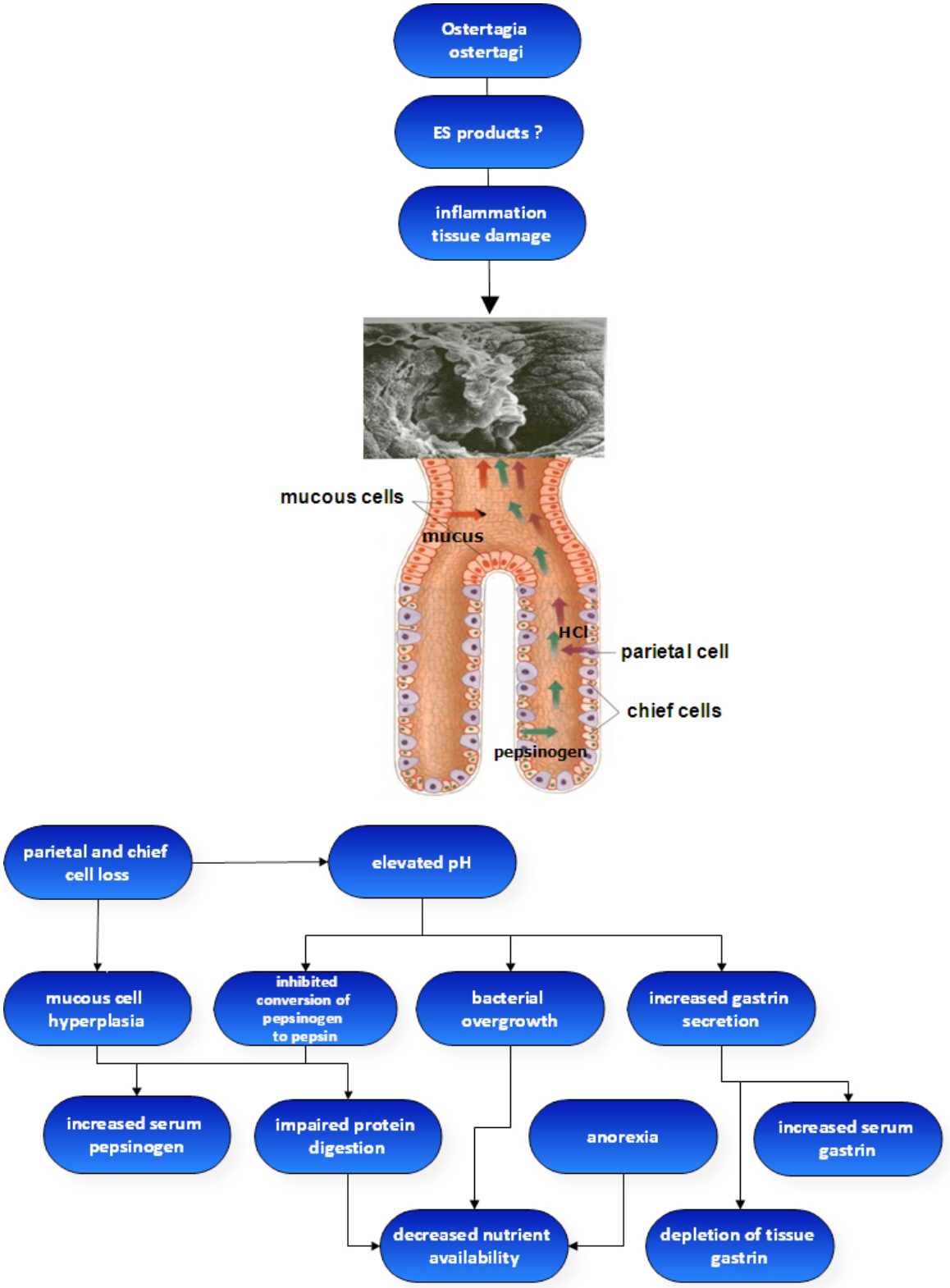


Figure 1.2 Overview of the pathogenesis during ostertagiosis. The development of *Ostertagia* from L3 to adult worms causes inflammation and tissue damage (parietal and chief cell loss). This induces a cascade of important morphological, physiological and biochemical changes which are described in the bottom part of the figure. Supposedly, excretory-secretory products (ESPs) may play a key role in these processes.

2.1. Abomasal pH

Changes in abomasal pH rapidly occur during an *Ostertagia* infection. This reduction in gastric acid secretion can be attributed to the loss of functional activity of parietal cells. As L3 larvae penetrate and young adults emerge from the gastric glands, the secreting cells lining the gland (parietal and zymogen cells) are damaged and replaced by immature cells with reduced secretory activity and lacking tight junctions (McKellar, 1993). Also the neighbouring glands are affected and lose their cellular differentiation. This parietal cell loss and typical signs of necrosis have been observed in several studies with *O. ostertagi* (Fox, 1993) and the closely related sheep parasite *T. circumcincta (*formerly known as *Ostertagia circumcincta)* (Scott *et al*., 1998b; 2000).

Parietal cells may be the primary target of both *Ostertagia* sp. and *Haemonchus*, since both parasites showed accelerated mortality at increasing acidity *in vitro* (Haag, 1995; Lawton *et al*., 2002) and *in vivo* (Simpson *et al*, 1999). These raised pH levels could be indispensable for creating a favourable environment for parasite survival. However the duration and extent of decreased acidity is largely dependent on worm burdens and abomasal secretory capacity and these may vary significantly within the animals and certainly between studies. Adult worms may create a favourable microenvironment in the mucus layer and thereby leaving the pH of abomasal content unaffected. This could explain the contradictory results from McKellar *et al.* (1987) who failed to notice a rise in pH, throughout the trial, in calves infected by surgical transplantation of 9000 adult *Ostertagia*. Furthermore, both Simpson *et al.* (1997) and Scott *et al*. (2000) detected in a few sheep only a moderately rise of pH within 2-3 days after transplant of respectively adult *H. contortus and T. circumcincta.*

Host immune responses to parasitism may also contribute to inhibition of acid secretion. The oxidative bursts of eosinophils and neutrophils are aspecific defence mechanisms and therefore may damage both the parasite and the host tissue (Scott *et al*., 2000; Merkelbach *et al*., 2002). This phenomenon has also been observed during *Helicobacter pylori* infections. Granulocyte cytokines like interleukin-1 β (IL-1β) and Tumour Necrosis Factor-α can inhibit the gastric acid secretion (Beales and Calam, 1998). Moreover, Yasunaga *et a*l. (1997) have reported a correlation between the mucosal IL-1β levels and the severity of parietal cell dysfunction during *H. pylori* induced gastritis.

2.2. Gastrin secretion

Infection with *Ostertagia* in ruminants is also characterised by hypergastrinaemia (Fox, 1997; Hertzberg *et al*., 2000; Fox *et al*, 2006). Gastrin stimulates the abomasal acid and pepsinogen secretion, inhibits gut motility and possesses a trophic activity on the fundic mucosa (Hoste, 2001). Gastrin exerts its activities through direct stimulation of the parietal cells but mainly through

histamine release from the enterochromaffin-like (ECL) cells. Gastrin is principally secreted by antral G cells as a precursor molecule and is processed to 2 biologically active peptides G34-NH_2 and G17-NH_2, which are C-terminal amidated.

The elevated pH levels appear to be the main stimulus for these higher gastrin concentrations in sheep or cattle infected with *Ostertagia* sp. (Fox *et al*., 2002, 2006). A significant correlation was observed between acid secretion and blood total gastrin concentrations. Furthermore, the circulating gastrin concentrations were largely comprised of gastrin-34 (G-34) in parasitized animals while the gastrin-17 (G-17) remained unaltered (Fox *et al*., 1993; Purewal *et al*., 1997). These elevated G34 levels can be explained by a marked rise in gastrin demands by the host that cannot be fulfilled by G17-NH2 alone resulting in incompletely processed molecules like G 34 or G17-gly (Fox *et al*., 2006; Purewal *et al*., 1997). In contrast, Fox *et al*. (2002, 2006) observed hypergastrinaemia during the early stages of infection despite low pH levels in the abomasum. This supported another theory in which a role for parasitic secretions was implicated in the aetiology of elevated gastrin levels. Anderson *et al*. (1981) also observed the increased gastrin concentration occurring before the elevated abomasal pH after transfer of larval or adult *T. circumcincta*. Also in cattle, it was suggested that physical or chemical stimuli from the parasite could initiate the hypergastrinaemia in absence of altered abomasal pH (McKellar *et al*., 1987). The parasite ESPs may activate the G-cells directly or initiate the release of inflammatory mediators like histamine (Bado *et al*., 1994) or TNF (Lehmann *et al*., 1996), known stimulants of gastrin secretion. In contrast, Lawton *et al.* (2002) reported that ESPs from *T. circumcincta* were unable to consistently stimulate ovine antral mucosal preparations.

A significant decrease in both pyloric tissue gastrin levels and in gastrin-producing G cells was observed by Fox *et al.* (1993) during an infection with *O. ostertagi*. Purewal *et al*. (1997) suggested that depletion of previously stored gastrin reserves contributed only in a small manner to elevated blood gastrin concentrations. Instead, the authors detected an 11-fold increase in gastrin mRNA transcription which might indicate an increase in peptide synthesis in the remaining G cells and could therefore make significant contributions to hypergastrinaemia. Reduced gastrin turn-over or clearance rates have also been implicated in the altered blood gastrin levels (Purewal *et al*., 1997).

Several publications suggested an association between this hypergastrinaemia and reduced appetite in *O. ostertagi* infected cattle (Fox *et al*., 1989a; 1989b; 2002). One hypothesis is that this happens through leptin. This peptide is produced by adipocytes, the placenta and gastric epithelial cells and plays a key role regulating food intake, body weight and fat mass (Guilmeau *et al*., 2004; Fox *et al*., 2006). Parasite-induced hypergastrinaemia may stimulate the leptin expression through activation of gastrin/CCK-B receptor on adipocytes and/or

gastric epithelial cells (Attoub *et al*., 1999; Fox *et al*., 2006). Increased blood levels of leptin would then down-regulate the neuropeptide-Y (NPY) synthesis, a neuronal peptide which is responsible for central control of the feeding behaviour. This results in reduced feed intake and mobilisation of the adipose tissue energy reserves. However, this pathway was recently questioned by Fox *et al*. (2006). Neither vagotomy and/or ganglionectomy of *T. circumcincta* infected animals resulted in a changed feeding pattern compared to infected animals without surgery. They could not provide evidence for a significant association between feed intake, central nervous system, leptin and gastrin blood levels. Therefore other mediators like IL-1β (Wisse *et al*., 2004), TNF-α (Elsasser *et al*., 1998) and ghrelin (Lippl *et al*., 2004) have been implicated in the regulation of appetite control.

2.3. Pepsinogen secretion

Jennings *et al*. (1966) postulated that the hyperpepsinogenaemia was caused by the emergence of L5 larvae from the gastric glands. The gastric glands are stretched and damaged and finally replaced by immature undifferentiated cells without tight junctions. The increase of interstitial pepsinogen cannot only be attributed to increased mucosal permeability (leakage). Another important contribution to hyperpepsinogenaemia originates in the impaired pepsinogen conversion to pepsin, due to rise in abomasal pH. In type-2 ostertagiosis, additional factors like increased capillary permeability or surface are necessary to explain hyperpepsinogenaemia (Baker *et al*., 1993). Stringfellow and Madden (1979) assessed the difference in mucosal permeability between infected and non-infected calves but detected no difference. Instead, they delivered ultrastructural evidence that pepsinogen was secreted directly into the bloodstream. In another study, it was observed that plasma pepsinogen levels were elevated within 24-48 hours after transfer of adult *Ostertagia* in abomasum of parasite naïve sheep and calves (McKellar *et al*., 1986; Scott *et al*., 2000). These results contradict the assumption that the emergence of L5s is responsible for increased mucosal permeability and thus elevated pepsinogen levels.

Immunohistochemical studies revealed another aspect of the hyperpepsinogenaemia during nematode infections (Scott *et al*., 1999, 2000). Hyperplasia is one of the typical morphological changes observed during infections with the *Ostertagia* sp. in cattle and sheep but also in *H. contortus* infected sheep. Two types of hyperplasia have been reported: 1) a nodular type located in the vicinity of parasitized glands and mainly seen in *O. ostertagi* infected animals; 2) a more generalised diffuse type caused by *H. contortus* (Scott *et al*., 1998a) and *T. circumcincta* (Scott *et al*., 1998b; Scott and McKellar, 1998). Both types are characterised by increased numbers of mucous cells that secrete both mucus and pepsinogen. These hyperplastic regions produce more pepsinogen compared to the adjacent mucosa and may alter the

plasma pepsinogen concentrations. As mentioned above, hyperplasia is regulated by blood gastrin and can be considered as a local adaptation process by the host in order to maintain the enzymatic function of gastric epithelia. Parasite mediators themselves may influence the epithelial cells or induce a hypersensitivity reaction with accumulation of eosinophils and mast cells (Baker *et al*., 1993; Wildblood *et al*., 2005). Leukotrienes may also stimulate pepsinogen secretions (Serrano *et al*., 1997).

2.4. Gastro-intestinal motility

A study of Fox *et al*. (1989a) demonstrated a reduced rate of passage of ingesta in calves exposed to a trickle infection with *O. ostertagi.* Similar studies in sheep infected with *Trichostrongylus axei* (Bueno *et al*., 1975) and *H. contortus* (Bueno *et al.*, 1982) showed remarkable disturbance in gastrointestinal motility. Alternating circulating levels of gastrointestinal hormones has been linked to these gut motility disturbance. For example, hypergastrinaemia has been implicated in several effects of gastrointestinal parasitism amongst which increased depth of abomasal mucosa, altered gut motor functions and reduced feed intake (Hoste, 2001).

In contrast, hypercontractility of the GI tract has been observed during experimental *Trichinella spiralis* infections in murine hosts. These infections have been employed extensively as a laboratory model to study various GI illnesses. *T. spiralis* inhabits the (sub)mucosal layers of the proximal small intestine (duodenum and jejunum) and causes an inflammatory response accompanied with altered GI motility, comparable to disorders like inflammatory bowel syndrome (Khan and Collins, 2004; Tanovic *et al*., 2006; Venkova and Greenwood-Van Meerveld, 2006). These authors demonstrated that inflammation of the gastrointestinal tract (Th2 cytokine responses) could account for hypercontractility in the upstream infected regions while the more distal parts of the intestine exhibit reduced contractility. A propulsive movement is created in the intestine and could thereby promote expulsion of the parasite. However, it is unclear if an *Ostertagia* infection induces a similar response and rapid expulsion of *Ostertagia* has not been reported up till now (Hoste, 2001).

3. Role of parasite excretory-secretory material in the pathogenesis

As described above, the development of *Ostertagia* from L3 to adult worms is associated with several important morphological, physiological and biochemical changes in the abomasal mucosa (McKellar, 1993). Although the mechanisms used by the parasites to change the abomasal environment are largely unknown, an important role has been attributed to parasite ESPs.

3.1. Introduction / state of the art

ESPs are comprised of the collective material released by the parasites *in vitro* and presumably also *in vivo*. These ESPs are isolated from culture supernatants of L3, L4 and adult nematodes during several days (Geldhof *et al*., 2000). ESPs are often stage specific and may be derived from the parasite surface, from specialized secretory glands or as by-products of parasite digestion. However, somatic proteins released by deteriorating parasites *in vitro* may also be present. ESPs are thought to play an important role in host penetration, parasite feeding but also take part in parasite-mediated immunosuppression (Klesius, 1993; reviewed by Knox, 2000). They may also be implied in the pathophysiological changes in the abomasum during *Ostertagia* infection.

A role for parasite ESPs in **elevated stomach pH** has been implicated by several authors based on the rapid reduction of acid secretion after adult worm transfer and the equally rapid recovery after anthelmintic treatment (Anderson *et al*., 1985; Simpson *et al.*, 1999; Scott *et al*., 2000). The participation of ESPs was assessed during *in vivo* experiments. A first study by Eiler *et al*. (1981) showed that intraperitoneal injection of *O. ostertagi* extracts blocked the hydrochloric acid secretion in rats. In a second trial, Simpson *et al*. (1999) transplanted a large number of adult *T. circumcincta* into the sheep abomasum while they were confined within a porous bag. This prevented direct contact of the parasite with the gastric mucosa. The abomasal pH was elevated soon after worm transfer and especially after feeding the sheep. Similar results with unrestrained worms indicate that the pH rise is not due to active chemicals released by dying or dead parasites in the porous bag. These observations suggested that ESPs are the initial trigger for altered abomasal pH rather than replacement of damaged parietal cells. Finally, an *in vivo* experiment in sheep infected with *Ostertagia leptospicularis* could demonstrate that the gastric acid secretion was blocked despite the presence of functional parietal cells and high serum gastrin levels which is normally a potent stimulator of acid secretion (Hertzberg *et al*., 2000). The authors suggested that parasite mediators may interfere directly or indirectly with the parietal cell function by an unknown mechanism. The indirect mechanism may act via reduced histamine release by

enterochromaffin-like (ECL) cells that normally stimulates the parietal cell (Hertzberg *et al*., 1999; 2000). *In vitro* experiments provided additional information that favours the hypothesis of ESPs involvement in parietal cell dysfunction. ESPs of *H. contortus* have proven to contain inhibitors for acid secretion when they are incubated *in vitro* with dispersed rabbit gastric glands (Merkelbach *et al*., 2002). The active components of approximately 5000 MW caused increased vacuolisation of epithelial cell cultures and uptake of neutral red (NR), a specific marker for the vacuolar space (Huber *et al*., 2005). Cell detachment and increased cell death were also induced by the ESPs from adult worms.

To explain the link between GI parasites en hyperpepsinogenaemia, there are several theories. The adult worms may increase the mucosal permeability through the release of ESPs or damage the epithelial cells while penetrating the mucosal layer. On the other hand, the adult *Ostertagia* may directly stimulate **the pepsinogen secretion** by zymogen cells. This was confirmed by McKellar *et al.* (1990a). They observed that isolated bovine and ovine parasite-naïve gastric glands released small amounts of pepsinogen under the influence of *Ostertagia* sp. ESPs. In a later study, *T. circumcincta* ESPs could stimulate pepsinogen secretion in isolated abomasal sheets from previously exposed animals while tissue preparations from parasite-naïve sheep did not respond. A hypersensitivity-like reaction may be the most reasonable explanation for this phenomenon (Scott and McKellar, 1998).

Scott and McKellar (1998) assessed the influence of *T. circumcincta* ESPs on the **abomasal motility** *in vitro*. Smooth muscle strips of previously infected animals consistently contracted in response to ESPs, while strips from parasite-naïve animals showed no reactivity. These data suggest a host–parasite (ESPs) interaction in the previously infected animals. However, specific ESPs involved in this process need to be determined further and although nematode cholinesterases may also influence gut motility, this seems unlikely in *Ostertagia* infections. *Ostertagia* cholinesterases appear to be restricted to the parasite rather than being secreted into the gut lumen like the *Trichostrongylus* enzymes (McKellar, 1993).

Immunity against *Ostertagia* develops slowly and is usually incomplete (Gasbarre, 1997; Gasbarre *et al*., 2001). Increasing evidence has shown that *Ostertagia* can modulate the host immune response by releasing ESPs: they exhibit next to antigenic properties also regulatory activities (Yang *et al.*, 1993). Several potential suppressive mechanisms have been proposed amongst which generation of suppressor T cells (Klesius *et al*., 1984), polyclonal lymphocyte activation (Gasbarre, 1997), the release of parasite products that modulate cell growth (De Marez *et al*., 2000) and non-specific suppression of peripheral blood lymphocyte responsiveness to mitogens (Klesius *et al*., 1984; Snider *et al*., 1986; Cross and Klesius, 1989). Recently, Gomez-Munoz *et al*. (2004) assessed

the inhibitory activity of *O. ostertagi* ESPs. ESPs from the L4 stage were capable of inhibiting the growth of lymphocytes after stimulation with Concanavalin (Con A), a T cell mitogen.

On the other hand, both *Ostertagia* L3s and their ESPs possess chemotactic activity for eosinophils (Washburn and Klesius, 1984; Klesius *et al*., 1986). Klesius *et al*. (1989) also visualized the presence of an eosinophil chemotactic factor (ECF) in *Ostertagia* L5 organelles that was identified as a lectin component (Klesius, 1993). The authors suggested that this chemoattractant may be present in all larval stages and perhaps even in adults and may contribute to the eosinophilia observed during ostertagiosis. A more recent experiment demonstrated a similar activity in whole worm extracts from L3s, L4s and adult *T. circumcincta* but also in adult *H. contortus*. ES material from *Teladorsagia* L3 larvae possessed the same chemotactic activity. By means of an adapted assay, it was demonstrated that live L3 larvae from *T. circumcincta* and *H. contortus* also could stimulate eosinophil migration. In contrast, this chemoattractant activity was not detectable in the free-living nematode *Caenorhabditis elegans*. This suggests a role for these immunomodulatory mechanisms in the pathogenesis of GI nematode infections (Wildblood *et al*., 2005).

3.2. Composition of *O. ostertagi* ES material

In the previous paragraphs, it has become clear that *Ostertagia* ESPs play an important role in the pathogenesis. However, specific parasite ESPs involved in this host-parasite interaction have not been defined. Therefore, we will give an overview of known *O. ostertagi* ESPs, as described by Vercauteren *et al*. (2003). In order to identify and unravel the composition of *O. ostertagi* ESPs, *Ostertagia* cDNA libraries were immunoscreened with polyclonal rabbit serum raised against *in vitro* ESPs of L3s, L4s and adults. 41 ES proteins were identified, of which 9 were novel. Homologues of structural proteins (actin, kinesin and vitellogenin), metabolic enzymes, stress-proteins (heat shock protein), antioxidantia (thioredoxin peroxidase) and probably also somatic proteins, non-specifically released by disintegrating nematodes, were present in the pool of immunoreactive proteins. Therefore, the authenticity of the recognised ES proteins was evaluated based on two criteria. Monospecific antibodies against the 41 clones were purified from a pool of anti-ES antibodies by affinity selection and used to develop Western Blots of ESP and extracts. The presence of a signal peptide also suggested that these proteins are genuinely secreted. As a result of both selection strategies, 15 cDNA clones were considered to code for authentic secreted proteins. The ESPs are described in table 1.1. Finally, a paragraph dedicated to parasite proteases is also included in this chapter. These ESPs have been studied extensively both in *Ostertagia* and in other nematodes and appear to have a crucial role in the host-parasite homeostasis.

Table 1.1 Characteristics of 15 putatively secreted ESPs* of *O. ostertagi* (Vercauteren *et al*., 2003).(* experimental evidence present)

Stage	Homology	Species	Possible function	Accession number
Ad	Activation associated secreted protein (ASP) 1*	*A. caninum*	Associated with transition to parasitism	AJ.310813
L3 L4-Ad	Disulphide isomerase precursor*	*S. mansoni* *O. ostertagi*	Catalyses the formation of disulfide bonds and associated folding in the secretory protein biosynthesis	AJ318479 AJ318480 AJ419174
Ad	Heat-shock protein*	*N. brasiliensis*	Molecule chaperone Induced by various stress factors	AJ310811 AJ318799
L4-Ad	Nematode polyprotein allergen*	*D. viviparus*	Binds fatty acids and retinol Involved in distribution of lipids and in the modification of host tissue environment	Z46800
L4	Globin-like host-protective antigen*	*T. colubriformis*	Oxygen transport	AJ427357
L3	None*	None	?	AJ318472
L3	None*	None	?	AJ318473
Ad	Similar to K07A1.10*	*C. elegans*	?	AJ318791
Ad	L4 cDNA clone*	*O. ostertagi*	?	AJ427476
L3	Dermal gland protein	*X. laevus*	Growth factor in the germinal layer of the epidermis Protection of the skin from external environment	AJ318474 AJ318475
L3	ATP synthase F0 subunit 6*	*C. elegans*	Couples proton movement and synthesis of ATP	AJ318476
L4	Similar to F02A9.2	*C. elegans*	Retinol- binding protein	AJ427358
Ad	Vitellogenin 6	*C. elegans*	Major component of egg yolk Supply growing	AJ310819
Ad	SXC-1 protein*	*O. ostertagi*	Protein-protein interactions in surface coat proteins signalling ligand	AJ310810
Ad	Similar to JC8.8*	*C.elegans*	?	AJ318795

3.2.1. Activation associated secreted proteins

Activation-associated secreted proteins (ASP) are the most abundant antigens present in adult *O. ostertagi* ES (Geldhof *et al*., 2003b) and have been identified in ESPs of various other nematodes such as *Ancylostoma caninum* (Moser *et al.*, 2005; Hawdon *et al*., 1999), *A. duodenale* (Bin *et al*., 1999), *A. ceylaticum* (Goud *et al*., 2004), *Necator americanus* (Daub *et al*., 2000; Asojo *et al*. 2005), *Onchocerca volvulus* (Tawe *et al*., 2000), *Brugia malawi* (Murray *et al*., 2001) and *H. contortus* (Schallig *et al*., 1997a,b; Bethony *et al*., 2006). They are members of a nematode specific protein family belonging to the SCP/Tpx-1/Ag5/PR-1/Sc7 (Pfam PF00188) family, which is found in a broad evolutionary spectrum of proteins, ranging from plants to mammals.

The nematode ASPs may be divided in 3 types: two-domain and single domain ASPs showing homology to either the C-terminal or N-terminal domain of the two-domain ASP. The 2-domain and the C-type single domain ASPs were first discovered in *H. contortus* (Hc40 and Hc24) and *A. caninum* (ASP1 and ASP2) (Hawdon *et al*., 1996, 1999; Schallig *et al*., 1997b; Rehman and Jasmer, 1998) and more recently in *O. ostertagi* (Visser *et al*., 2007). Up till now, N-type single domain ASPs have only been described in *O. ostertagi* (Oo-ASP1 and Oo-ASP2) and *C. punctata* (Yatsuda *et al*., 2002; Geldhof *et al*., 2003b). Monospecific antibodies, raised against Oo-ASP1, were used for probing Western blots of extract and ES material of all three life stages (Geldhof *et al*., 2003b). The strongest expression of Oo-ASP1 and 2 was observed in adult ESPs and to a lesser extent in adult extract. A less prominent band was visible in L4 extract and ESPs while no specific reaction was detected in the L3 stage. The transcription patterns of *Oo-asp1*, *Oo-asp2* and 13 other *Ostertagia* ASPs were evaluated by semi-quantitative PCR or Real-time PCR (Visser *et al.*, 2007). The majority of the ASPs was highly transcribed in the L4 larval stage, whereas others (including *Oo-asp1* and *Oo-asp2*) were highly enriched in adult male worms. The low transcription levels in the *Ostertagia* L3 stage, both infective and exsheathed, did not correspond with the high transcription levels of ASPs in *A. caninum*, *O. volvulus* and *B. malayi* larvae (Tawe *et al.*, 2000; Murray *et al*., 2001). By immunohistochemistry, the native Oo-ASPs 1 and 2 were localised in some parts of the reproductive tract from the female parasite while the two-domain Oo24 mainly was localized in the oesophagus.

The function of the nematode ASPs is still unknown. It has been postulated that nematodes may require a host trigger for ASP transcription initiation such as transition to parasitism (Murray *et al*., 2001; Moser *et al*., 2005; Hawdon *et al*., 1996, 1999). Real-time PCR data supported this theory in *Ostertagia* because exsheathing L3 larvae *in vitro* induced a 10-fold and 4-fold rise of the Oo-ASP1 and Oo-ASP2 transcription levels, respectively, within a few hours. However, Visser *et al*. (2007), revealed a strong male enriched transcription pattern for 9 *O. ostertagi* ASPs which suggests a role in reproduction or in the development of

the reproductive system. This phenomenon has also been observed in *C. elegans*, *Trichostrongylus vitrinus*, *Oesophagostomum dentatum* and *B. malayi* (Reinke *et al*., 2004; Nisbet and Gasser, 2004; Li *et al*., 2005; Cottee *et al*., 2006). A role in sperm maturation and fertilization has been reported in several other members of the SCP/Tpx-1/Ag5/PR-1/Sc7 family amongst which TPX, AEG1, CRISP-1 and CRISP-2 (Kasahara *et al*., 1989; Mizuki and Kasahara, 1992, Mizuki *et al*., 1992; Krätzschmar *et al*., 1996). However, the Oo24 immunolocalisation data in the pharynx and the abundant excretion/secretion of ASPs contradict this reproduction hypothesis. The male enrichment on transcriptional level of 9 ASPs could indicate the existence of an ancestral ASP molecule with an important function in male reproduction. It is possible that the current function is totally different and more situated in transition to parasitism.

Furthermore, ASPs have shown their protective capacity in multiple vaccination trials against the *Ancylostoma* species, *H. contortus* and *O. ostertagi* (Schallig *et al*., 1997a; Vervelde *et al*., 2002; Geldhof *et al*., 2002, 2004; Goud *et al.*, 2004; Meyvis *et al*., 2007). For *O. ostertagi*, Geldhof *et al*. (2002, 2004) obtained an ESP fraction by thiol-sepharose chromatography of adult ESPs (ES-thiol), highly enriched of Oo-ASP1 and OoASP2, and evaluated their protective capacities in two independent vaccine trials. The significant protective immune response in cattle, immunised with ES-thiol and QuilA as adjuvant, was characterised by a reduction of the geometric mean cumulative egg counts between 56 and 62 %, stunted adult worms, more inhibited L4 larvae and a reduction of 18 % in total worm burden (Geldhof *et al*., 2002; 2004; Meyvis *et al*., 2007).

3.2.2. Protein disulphide isomerases

Protein disulphide isomerases (PDI) are multifunctional proteins, present in ESPs from *Dirofilaria immitis* (Chandrashekar *et al*., 1998), *T. circumcincta* (Craig *et al*., 2006; Martinez-Valladares *et al*., 2007) and *O. ostertagi* (Geldhof *et al*., 2003a; Vercauteren *et al*., 2003). Furthermore, PDIs have also been studied extensively in *A. caninum* (Epe *et al*., 1998) and *C. elegans* (Page, 1997; Myllyharju *et al*, 2002; Winter *et al*, 2007,) where a conserved family of 3 PDIs has been examined recently. PDIs are required for formation and isomerisation of disulphide bridges between cysteines and for transit through endoplasmic reticulum. They may also act as chaperones, forming the beta subunit of the prolyl-4-hydroxyprolylase (P4H) enzyme and triacylglycerol transfer proteins (TTP) (Martinez-Valladares *et al*., 2007). P4H is an $\alpha_2\beta_2$ tetramer that catalyses the formation of 4-hydroxyprolyl residues of the collagen chains while the heterodimer TTP facilitates the loading of apolipoproteine B with lipid (Freedman *et al*., 1994; Bradbury *et al*., 1999).

In *O. ostertagi* 2 types of PDIs have been discovered up till now: a PDI-like protein and genuine PDI enzymes. **Ost-PDI-1** (PDI-like protein) was described by Vercauteren *et al* (2003). The corresponding cDNA clone was isolated from the L3 larval cDNA library by means of immunoscreening with rabbit anti-L3 ES serum. Another *Ostertagia* PDI, **Ost-PDI-2**, was unexpectedly isolated by Geldhof *et al.* (2003a) when screening for antibody-degrading aspartyl proteases in ESPs of the L4 and adult life stages with a pepstatin A-agarose column. Recently, a *T. circumcincta* PDI (Tc-PDI) was described which shows 99% homology with the Ost-PDI 2 protein. Both have a molecular weight of 55 kDa and contain 2 thioredoxin boxes (catalytic centre), a N-terminal signal peptide and also a C-terminal endoplasmic reticulum retention sequence (HTEL). Western blots of extract and ESPs of the L3, L4 and adult *O. ostertagi* life stages, probed with anti-Ost-PDI-2 antibodies, demonstrated one prominent band at 55 kDa in all the extracts and in L4 and adult ESPs. This developmental expression pattern was confirmed by real time PCR. In somatic extracts of L3, L4 and adult *T. circumcincta* a similar band was detected with antibodies raised against Tc-PDI (Martinez-Valladares *et al.*, 2007).

Monospecific anti-Ost-PDI2 antibodies were used to detect the corresponding native protein on sections made from the different life stages of *O. ostertagi*. Strong immunofluorescent labelling could be detected in the hypodermis, the intestinal cells and the pharynx of L4s and adults. On the L3 sections, only the intestinal cells showed immunolabeling (Geldhof *et al*., 2003a). The hypodermal location in L4 and adult *O. ostertagi* corresponds with the *C. elegans* homologue. Mutations in this *C. elegans* pdi2 gene resulted in impaired collagen synthesis and hence severe defects in body morphology (cuticle) and embryonic lethality (Winter *et al*., 2007). However, the intestinal and pharyngeal immunolocalisation data and their excretory/secretory character suggest additional functions. It has been postulated that *A. caninum* PDI enzymes are released during tissue migration and skin penetration by infective larvae (Hotez *et al*., 1992; Epe *et al*., 1998).

Moreover, both Ost-PDI2 and Tc-PDI are strongly recognised by sera from infected animals, which suggest also *in vivo* release of PDI. A major spot was detected on 2D Western blot of L4 *O. ostertagi* extract, probed with pooled sera of animals naturally infected with *O. ostertagi* (Geldhof *et al*., 2003a). Serum IgAs of experimentally infected sheep strongly recognised PDI on a *T. circumcincta* lysate immunoblot (Martinez-Valladares *et al.*, 2007).

3.2.3. Heat shock protein

Small heat shock proteins (sHSP) belong to a family of low molecular weight polypeptides (12 to 43 kD), which have been identified in almost all eukaryotes. A sHSP has been identified in *T. circumcincta* L4 ESPs (Craig *et al*., 2006) and in *O. ostertagi* ESPs (Vercauteren *et al*., 2003; 2006). Additionally, they have been

described in *Nippostrongylus brasiliensis* (Tweedie *et al*., 1993), *D. immitis* (Lillibridge *et al*., 1996), *B. malayi* (Raghavan *et al.*, 1999) and *H. contortus* (Hartman *et al*., 2003). In *C. elegans*, 16 sHSPs have been identified amongst which a 16 kDa species with several members that are only expressed under stress conditions (Strayer et al., 2003). sHSPs all posses an α-crystallin domain, which enables them to form higher order structures (Lindquist, 1986). sHSPs enhance the survival of cells exposed to various kinds of stress amongst which heat shock, heavy metals, oxidative stress, treatments with anti-cancerous and apoptosis-inducing agents (Arrigo, 1998). They are considered to perform a chaperone function by binding to misfolded or denatured proteins and targeting these for refolding or for degradation.

Recently, an *O. ostertagi* sHSP of approximately 18 kDa (Oo-HSP18) was described by Vercauteren *et al.* (2006). This protein was closely related to the sHSPs of *H. contortus, N. brasiliensis* and *C. elegans*. Although sHSPs generally appear to be encoded by multiple genes (Tweedie *et al*., 1993; Thompson *et al*., 1996), Southern blotting revealed one hybridizing band at approximately 2,8 kb, suggesting that Oo-hsp18 is encoded by a single-copy gene. Similar results were also obtained in *H. contortus* (Hartmann *et al*., 2003).

Several small *hsps* of *Brugia* sp. and *N. brasiliensis* are developmentally regulated (Raghavan *et al.*, 1999; Tweedie *et al*., 1993). In case of *Oo-hsp18*, the transcription levels in adult worms were respectively 170-, 757- and 413-fold higher than the levels in L3s, exsheated L3s and L4. The developmental expression pattern of Oo-HSP18 on Western blots of L3, L4 and adult ESPs corresponded with these real time data from the L3, L4 and adults life stage. Tissue-specific expression of Oo-HSP18 was situated in the body muscle layer of L4 and adult *Ostertagia*, which was consistent with previous data of other nematode HSPs (Vercauteren *et al*., 2006). On the other hand, the *H. contortus hsp20* was constitutively transcribed and expressed throughout its life cycle. Moreover, the HSP20 protein was located in the intestine and reproductive organs (Hartman *et al*., 2003).

Common stressors like elevated temperatures (transition from pre- to parasitic life stages), H_2O_2 (host immune responses) and anthelmintics continuously challenge *O. ostertagi* during infection. Incubation of L3 larvae at 42 °C induced significant increases in *Oo-hsp18* mRNA levels. However, no increases were reported upon challenge with levamisole or H_2O_2, even in the presence of altered morphology and behavior of the nematodes (Vercauteren *et al*., 2006). No increases of the HSP20 protein levels were observed in *H. contortus* upon heat (12, 31, 37 and 42°C) stimulation. This different reaction pattern to heat stress may reflect the differences in host and/or niche (Hartman *et al*. 2003).

As mentioned above, sHSPs may fulfill a more generalized chaperone role within the cell. The length of the N-terminal region seems to be associated with

this function as they appear to be important for oligomerisation. In *C. elegans*, HSPs with short N-termini (≤25 AA) are devoid of a chaperone function (Kokke *et al*., 1998). Oo-HSP18 and HSP20 both posses a 55 AA N-terminal domain that may suggest a chaperone function. The high concentrations of Oo-HSP18 in ESPs might indicate that Oo-HSP18 is associated with other proteins for stabilization during excretion (Vercauteren *et al*., 2006). Furthermore, in parasitic nematodes, sHSPs appear to play a role in transition from free-living stages to the host (Jecock and Devany, 1992). The localization of Oo-HSP18 in the musculature of L4 and adults might imply an additional role in movement (Vercauteren *et al*., 2006). Supposedly, the high polymorphism between the N-termini of different nematode sHSPs reflects these various *in vivo* functions of sHSPs proteins (Leroux *et al*., 1997a,b).

Because baculovirus recombinant Oo-HSP18 was recognized by pooled sera of naturally immunized calves, a vaccination trial was setup with rOo-HSP18 to investigate its potential protective capacities against an Ostertagia challenge infection. Although both recombinant and native Oo-HSP18 were recognized by sera from vaccinated animals, the recombinant failed to stimulate protective immune responses. Instead, the geometric mean egg counts and worm burdens of the rOo-HSP18 vaccinated cattle were higher compared to the control animals. This could be attributed to the use of a recombinant Oo-HSP18 which may not elicit an appropriate immune response, or the native HSP which is not protective (Vercauteren *et al*., 2006).

3.2.4. Ostertagia polyprotein allergen

The ***Ostertagia* polyprotein allergen** (OPA), isolated from the L4 larval and adult cDNA libraries as described by Vercauteren *et al*. (2003), appeared to be the most abundant antigen in L3 ESPs but also present in adult ESPs. OPA showed strong homology with the nematode polyprotein allergens (NPA) of *Ascaris suum*, *D. viviparus* and *Toxacara canis*. All these NPA homologues are secreted by worms in culture, with the exception of *T. canis* (Kennedy *et al.*, 1989; McGibbon *et al.*, 1990; Poole *et al*., 1992). These proteins have been the target of strong IgE antibody responses and were therefore referred to as allergens. NPAs are composed of tandem repeats and are posttranslationally processed into multiple polypeptides of approximately 14 kDa. They are characterised by extremely non-polar binding sites which enables them to bind retinoids, important signalling molecules in several cellular processes, and fatty acids, the major energy reserves and cell membrane components. They can be considered as non-specific lipid-binding carrier molecules that function in the pseudocoelomic fluid and connective tissues of the nematode. Furthermore, additional functions have been suggested for NPA released by living nematodes: these excreted/secreted NPA could be involved in capturing important host lipids for the parasites. The parasites could thereby modulate local inflammation and

immune responses in the host tissues by sequestering small lipids amongst which leukotrienes, prostaglandin D2 and signaling lipids like retinol (Kennedy, 2000). Numerous studies have tried to unravel the binding properties and conformation of these NPA (Solovyova *et al*., 2003; Jordanova *et al*., 2005). A recombinant form of the *A. suum* As-NPA-1A subunit appeared to exist as a monomer in solution, contradicting previous data from Kennedy *et al.* (1995) who proposed a dimeric organisation (Solovyova *et al*., 2003). Moreover, it has become clear that NPAs do not require dimerisation for binding their ligands nor is the monomeric status affected by ligand binding. The monomers may be released in a diffuse way by the parasite through an excretory system (Solovyova *et al*., 2003). A striking feature of NPAs is their resilience against thermal denaturation. Temperatures higher than 90°C are required for denaturation.

Southern blot analyses of XbaI-digested and EcoRI-digested *Ostertagia* genomic DNA with the OPA probe (EMBL accession Z46800) revealed one major band located at 12 kb and two other bands at 3.8 and 1.8 kb (Vercauteren, *et al.* 2004). This suggests a single copy *opa* gene, as reported previously in *A. suum*, *D. immitis*, *Brugia* sp., *D. viviparus* and *C. elegans* (reviewed by Kennedy, 2000). Western blots of somatic extracts of L3, L4 and adult *O. ostertagi* were developed with monospecific anti-OPA antibodies and resulted in a recognition pattern with 3 bands, differing from each other by 14 kDa (14, 28 and 42 kDa respectively). On Western blots of L3, L4 and adult *Ostertagia* ESPs only one prominent band at 14 kDa was detected. Stage specific transcription was investigated by means of real-time PCR and confirmed the results of the Western blots. The highest levels of transcription were detected in the L3 stage, while the levels in L4s and adults were respectively two- and fourfold lower. Tissue sections of the parasite developed with the same monospecific anti-OPA showed fluorescent signals in the intestinal cells of L3s, in the cuticle of L4s and adults and in the hypodermis of adult worms. One could imply that OPA is released into the environment by direct secretion from the gut in L3 larvae or from the cuticle through the hypodermis from L4s and adults. Similar spatial organisation was observed for Di5, the OPA homologue in *D. immitis*, with immunostaining in the hypodermis and cuticle (Poole *et al*., 1992). Alternative localisations of NPAs have been reported in several parasites. The first discovered NPA, ABA-1 of *A. suum*, was detected at high concentrations in the pseudocoelomic fluid while *Ascaridia galli* Ag-NPA-1 was situated mainly in the inner hypodermis cells, oviduct epithelium and pseudocoelomic cavity (Jordanova *et al*., 2005). In *Brugia*, gp15/400 was present in the basal lamina surrounding the oesophagus and separating the hypodermal cord and somatic musculature (Selkirk *et al*., 1993).

Finally, their allergenicity combined with the absence of mammalian homologues rendered the NPAs as valid vaccination candidates. A vaccination trial of cattle with native OPA of *O. ostertagi* in combination with QuilA resulted

in protection against *Ostertagia* challenge infections. The geometric mean cumulative faecal egg counts in the nOPA-vaccinated animals were reduced by 60% compared to the counts in the control group during the whole trial. Moreover, both male and female adult worms in nOPA-vaccinated animals were significantly shorter than the worms in the control animals. Reduced faecal egg counts were significantly correlated with elevated IgG1 levels while the IgG2 titres were only negatively correlated with adult worm length. No accumulation of effector cells (mast cells, globular leukocytes, and eosinophils) was detected in the abomasal mucosa of the nOPA-vaccinated animals. The most surprising observation was the absence of higher IgE levels that may suggest that nOPA is not intrinsically allergenic. In contrast to the native antigen, recombinant OPA (only the C-terminal part) expressed in *Escherichia coli* did not stimulate any protection. The lack of post translational modifications like glycosylations may have attributed to this failure. Another possibility could be that a full-length OPA is necessary for a correct folding, conformation and thus protection (Vercauteren *et al.,* 2004).

3.2.5. Proteases

Another aspect of the host-parasite homeostasis is the release of **proteases** by the parasite. It appears that these ESPs have a crucial role in facilitating parasite survival by assistance in the tissue penetration, digestion of host tissue and evasion of host immune responses (reviewed by Tort *et al*., 1999; Dzik, 2006). This combined makes them attractive candidates for vaccine development and chemotherapy. Therefore, a lot of work has been focused on identifying and characterising these *in vitro* released (IVR) proteinases of *H. contortus* (Gamble and Mansfield, 1996; Karanu *et al*., 1993; Rhoads and Fetterer, 1995; Yatzuda *et al*., 2006), *T. circumcincta* (Young *et al.,* 1995; Redmond *et al*., 2006) and also *O. ostertagi* (De Cock *et al*., 1993; Geldhof *et al*., 2000; De Maere *et al*., 2005a; 2005b). Based on the composition of their active site, proteases are subdivided in four major classes: serine, aspartic, metallo- and cysteine proteases.

Geldhof *et al.* (2000) studied the pH optima, substrate and stage specificity of the *Ostertagia* IVR proteinases from exsheathed L3, L4 and adult *O. ostertagi* extensively. At alkaline pH gelatin, casein, fibrinogen and mucin were degraded by metallo- and serine proteinases in a stage specific manner. The fibrinolytic activity of metallo- and serine proteinases has also been reported in *T. circumcincta* (Young *et al*., 1995). In blood-feeding nematodes like *H. contortus* (Cox *et al*., 1990) and *T. vitrinus* (MacLennan *et al*., 1997) the metallo-proteinases play a role in inhibition of blood clothing although this seems unlikely in a non obligate blood-feeder like *Ostertagia*. On the other hand, the L3 stage metallo-proteinases could be an important tool in tissue penetration as reported in *A. caninum* (Williamson *et al*., 2006) and *H. contortus* (Gamble and Mansfield, 1996). A pH optimum of 8.5 could be in conflict with the acidic abomasal

environment. The parasite may create a favourable microenvironment in the mucus layer and thereby leaving the acidic pH of abomasal content unaffected (Young *et al*., 1995). Furthermore, it has been reported by several authors that a rise in abomasal pH rapidly occurs during an *Ostertagia* infection and an important role has been attributed to parasite ES material (Simpson *et al*., 1997; Scott *et al*., 2000).

Additionally, at acidic pH, aspartyl proteases and cathepsin L-like enzymes (cysteine proteases) from L4 and adult *O. ostertagi* hydrolysed a variety of blood-proteins such as fibrinogen, albumin, haemoglobin and IgG but also mucin. Both mucin and albumin are major components of the gastrointestinal mucus and therefore could represent an important food source for the parasite. The presence of an aspartyl proteinase which hydrolyses IgG, is clearly an asset for parasite survival. Digestion of local immunoglobulins and hence immune evasion could be one of the important enzymatic activities of *Ostertagia* proteinases (Murray and Smith, 1994; Tort *et al*., 1999; Dzik, 2006). To conclude, stage specific release combined with their substrate specificity (in pH-dependent manner) may indicate that *Ostertagia* proteinases perform specific functions during the parasitic phase in the abomasum (Geldhof *et al*, 2000).

Previous vaccination trials with purified native cysteine proteases have demonstrated their protective capacity against *Fasciola hepatica* (Wijffels *et al.*, 1994; Dalton *et al*., 1996) and *H. contortus* (Boisvenue *et al.*, 1992; Knox *et al*., 1999, 2005; Redmond and Knox, 2004). Recently, the *O. ostertagi* protective ES-thiol fraction (Geldhof *et al*., 2002; 2004) was subfractionated through Q-Sepharose anion exchange chromatography to determine which components were responsible for the induced protection (Meyvis *et al*., 2007). Three different subfractions were obtained and tested in a vaccination trial:1) an ASP fraction (as described earlier 3.1.1), 2) a cysteine protease fraction and 3) a rest fraction. During the two-month period of the trial, the geometric mean cumulative faecal egg counts (FEC) of the 3 groups were reduced significantly (70-80%) compared to the QuilA control group. More specific, 80% reduction of FEC was observed in the animals immunized with the cysteine protease fraction, which is the highest level of protection induced against *O. ostertagi* in cattle published to date. Nevertheless, a non protective membrane bound *Ostertagia* S3-thiol fraction also contained cysteine proteases (Geldhof *et al*., 2002), but it is possible that these cysteine proteases differ from those present in ES-thiol. Furthermore, additional (potentially protective) proteins may be present in the cysteine protease fraction. Further purification of this protein fraction is therefore essential (Meyvis *et al*., 2007).

3.2.6. Globin

An 18 kDa globin-like antigen from ESPs of *T. colubriformis* was capable to stimulate a protective immune response in guinea pigs (Frenkel *et al*., 1992). Other globin-like ESPs have been reported in *T. circumcincta* (Craig *et al*., 2006), in *H. contortus* (Yatsuda *et al*., 2003) and *O. ostertagi* (Vercauteren et al., 2003). Two globin isoforms are present in strongylid nematodes: (1) a monomeric myoglobin-like isoform, located intracellular in the body wall and pharyngeal musculature and (2) a secreted, tetrameric globin in the cuticle (Blaxter, 1993). Western blots probed with an anti-globin rabbit serum Rb94 revealed one prominent band of 17 kDa in extracts of *O. ostertagi* adults and L4s (de Graaf *et al.*, 1996). Similar stage specificity was observed in *T. colubriformis* (Frenkel *et al*., 1992), *N. brasiliensis* (Blaxter *et al*., 1994), *A. suum* (Barret and Brophy, 2000) and *H. contortus* (Fetterer *et al*., 1999; Craig *et al*., 2006). This *Ostertagia* globin-like antigen (OoAdGlb) was purified from total adult worm extracts by liquid chromatography. OoAdGlb has a predicted molecular weigth of 36 kDa under non-reducing conditions, suggesting dimerisation of two 17kDa monomers (de Graaf *et al*., 1996). Both adult *H. contortus* globin molecules and *A. suum* myoglobin form dimers (Fetterer *et al*., 1999; Blaxter *et al*., 1994). Immunolocalization revealed the presence of this globin-like antigen in the body wall musculature and/or the cuticle of *O. ostertagi* adults. The developmentally and spatially regulated expression of globin–like antigens could represent a local adaptation to the micro-aerobic environment in the host intestine (Claerebout *et al*., 2005).

Suggested functions of nematode myoglobin and cuticular globins are oxygen transport, osmotic regulation, iron storage and oxygen detoxification (Blaxter, 1993). A remarkable feature of the secreted (cuticular) form of globin is their higher affinity for oxygen compared to the host globins and suggests an essential role for these globins in oxygen transport for muscular activity (Yatsuda *et al*., 2003). These are crucial factors for parasite survival, which makes globins attractive vaccine candidates. Therefore, the protective capacity of an adult stage *O. ostertagi* globin antigen was tested in four vaccination experiments in cattle. In a preliminary experiment, calves were immunized intraperitoneally with globin (in Freund's adjuvant) and challenged with an experimental trickle infection. This was followed by three subsequent field studies where calves were vaccinated intramuscularly with globin (in Quil A) and challenged with a natural gastrointestinal nematode infection on pasture. In all vaccine trials, elevated globin-specific antibody levels were detected in the vaccinated calves compared to the control animals. Only in the preliminary experiment (artificial challenge) and in the first field trial partial protection against reinfection was obtained with reduced cumulative faecal egg counts of 52-63% in the vaccinated calves. This strong variability in protection levels between the four vaccination trials may be attributed to several components: intramuscular versus intraperitoneal

immunization, age unresponsiveness and differences between globin batches. However, the inconsistent results cannot solely be explained by these suggestions (Claerebout *et al*., 2005).

3.2.7. Other ES proteins

Vercauteren *et al*. (2003) also discovered 10 other cDNAs that supposedly code for genuine secreted proteins (Table 1.1). Two L3 ES antigens (AJ318472, AJ318473) showed no homology to any known protein or ESTs in the public databases. Two ES antigens of adult parasites showed homology to a hypothetical *C. elegans* protein K07A1.10 (AJ318791) and an *Ostertagia* L4 cDNA (AJ427476). It was impossible to assign a possible function to the ESPs mentioned above.

A homologue of the dermal gland protein of *Xenopus laevis* was detected. This protein may possibly have a growth factor activity in the germinal layer of the epidermis and may also be involved in growth of regenerating glands and in protection of the skin from the external environment (Hauser *et al*., 1992).

An ESP from the L3 larval stage showed similarity with ATP synthase, a bipartite complex protein that is composed of an integral membrane proton channel (F_0) and a peripheral catalytic domain (F_1). The ATP synthase uses the energy of a proton gradient across the mitochondrial inner membrane for ATP synthesis. Depending on the species, mitochondrial ATPases are comprised of 10–16 subunits encoded by nuclear DNA while other subunits 6 & 8 are encoded by mitochondrial DNA. ATPase subunit 6 might be involved in the proton translocation through the membrane sector of the ATP synthase. Their role in the host-parasite interaction has not been defined yet.

The sole remaining L4 ES antigen (AJ427358) has significant similarity to a hypothetical 20.1 kDa protein (F02A9.2) of *C. elegans*. Furthermore, the hypothetical *C. elegans* protein showed homology to an immunodominant antigen of *Onchocerca volvulus* (Ov20) that belongs to a new class of small helix-rich retinol-binding proteins.

Significant hits with vitellogenin (AJ310819) were identified. Vitellogenins are large proteins (up to 700 kDa) and are stored as the major component of the egg yolk of almost all oviparous animals. Vitellogenins are synthesized by extra-ovarian tissues, secreted into the circulatory system, and subsequently internalized by developing oocytes through receptor-mediated endocytosis. Their main function is supplying growing embryos with amino acids. *C. elegans* has six vitellogenin genes, four of which are expressed (Chen *et al*., 1997).

The six-cysteine domains (36-amino acid long) were originally termed the NC6 (nematode-six-cysteine) motif and are comprised of CxD(4x)C(6x)C(12x)CxxTCxxC cassettes with 6 cysteine residues at distinct positions. This motif is fused with the N- and C-terminus of a diverse range of protein domains, such as surface coat proteins of *Toxocara canis* (Loukas *et al*., 2000) but also tyrosinases, zinc metalloproteases and some mucin-like proteins in *C.elegans* (Blaxter, 1998). In general, SXC motif containing proteins have a putative secretory signal peptide and are presumably secreted.

Finally, the *O. ostertagi* ESP *(*AJ318795) with homology to the hypothetical protein JC8.8 of *C.elegans* was identified as a transthyretin-like protein. This ESP will be discussed thoroughly in chapter 4 (Transthyretin-like proteins).

4.Conclusions

It has been shown that the bovine abomasal parasite, *O. ostertagi*, drastically modulates its environment, causing epithelial cell damage, accumulation of inflammatory cells and pH changes in the stomach during the infection. An important role in these processes has been attributed to parasite ESPs. Although the pathogenesis of ostertagiosis has been studied extensively, the underlying molecular, biochemical, immunological and physiological mechanisms of the host-parasite interaction have not been characterised in detail. Thus, more knowledge is required on the protein-protein interactions between *O. ostertagi* (ESPs) and cattle (abomasum) which are involved in the pathophysiological and/or immunological changes in the abomasum. Identification of interacting ESPs could open new perspectives for vaccination research and chemotherapy.

5. References

Anderson, N., Handsky, J. and Titchen, D.A. (1981). Effects of *Ostertagia circumcinta* infections on plasma gastrin in sheep. Parasitol 82, 401-410.

Anderson, N., Handsky, J. and Titchen, D.A. (1985). Effects on plasma pepsinogen, gastrin and pancreatic polypeptide of *Ostertagia* spp. transferred directly into the abomasum of sheep. Int J Parasitol 15, 159-165.

Arrigo, A.P. (1998). Small stress proteins: chaperones that act as regulators of intracellular redox state and programmed cell death. Biol Chem 379, 19-26.

Asojo, O.A., Goud, G., Dhar, K., Loukas, A., Zhan, B., Deumic, V., Liu, S., Borgstahl, G.E. and Hotez, P.J. (2005). X-ray structure of Na-ASP-2, a pathogenesis-related-1 protein from the nematode parasite, *Necator americanus*, and a vaccine antigen for human hookworm infection. J Mol Biol 346, 801-14.

Attoub, S., Levasseur, S., Buyse, M., Goiot, H., Laigneau, J.P., Moizo, L., Hervatin, F., Le Marchand-Brustel, Y., Lewin, J.M. and Bado A. (1999). Physiological role of cholecystokinin B/gastrin receptor in leptin secretion. Endocrinol 140, 4406-10.

Bado, A., Moizo, L., Laigneau, J-P., Delwaide, J. and Lewin, M.J.M. (1994). H3-receptor regulation of vascular gastrin and somatostatin releases by the isolated rat stomach. Yale J Biol Med 67, 113-21.

Baker, D.G., Bruss, M.L. and Gershwin, L.J. (1993). Abomasal interstitial fluid-to-blood concentration gradient of pepsinogen in calves with type-1 and type-2 ostertagiosis. Am J Vet Res 54, 1294-8.

Barret, J. and Brophy, P.M. (2000). Ascaris haemoglobin: new tricks for an old protein. Parasitol Today 16, 90-91.

Beales, I.L.P. and Calam, J. (1998). Interleukin 1β and tumor necrosis factor α inhibit acid secretion in cultured rabbit parietal cells by multiple pathways. Gut 42, 227-234.

Bethony, J.M., Loukas, A., Hotez, P.J. and Knox D.P. (2006). Vaccines against blood-feeding nematodes of humans and livestock. Parasitol 133, Suppl:S63-79.

Bin, Z., Hawdon, J., Qiang, S., Hainan, R., Huiqing, Q., Wei, H., Shu-Hua, X., Tiehua, L., Xing, G., Zheng, F. and Hotez, P. (1999). *Ancylostoma* secreted protein 1 (ASP-1) homologues in human hookworms. Mol Biochem Parasitol 98, 143-9.

Blaxter, M.L. (1993). Nemoglobins: divergent nematode globins. Parasitol Today 9, 353 360.

Blaxter, M.L., Ingram, L. and Tweedie, S.(1994). Sequence, expression and evolution of the globins of the parasitic nematode *Nippostrongylus brasiliensis*. Mol Biochem Parasitol 68, 1-14.

Blaxter, M. (1998). *Caenorhabditis elegans* is a Nematode. Sci 282, 2041–2046.

Bradbury, P., Mann, C.J., Köchl, S., Anderson, T.A., Chester, S.A., Hancock, J.M., Ritchie, P.J., Amey, J., Harrison, G.B., Levitt, D.G., Banaszak, L.J., Scott, J. and Shoulders, C.C. (1999). A common binding site on the microsomal triglyceride transfer protein for apolipoprotein B and protein disulfide isomerase. J Biol Chem. 274, 3159-64.

Boisvenue, R. J., Stiff, M. I., Tonkinson, L. V., Cox, G., N. and Hageman, R. (1992). Fibrinogen-degrading proteins from *Haemonchus contortus* used to vaccinate sheep. Am J Vet Res 53, 1263-1265.

Bueno, L. Dorchies, P. and Ruckebusch, Y. (1975). Analyse electromyographique des perturbations motricees lies aux strongyloses gastro-intestinales chez les ovins. C.R. Seances. Soc Biol Paris 169, 1627-1632.

Bueno, L., Dakkak, A. and Fioramonti, J. (1982). Gastro-duodenal motor and transit disturbances associated with *Haemonchus contortus* infection in sheep. Parasitol 84, 367-374.

Chandrashekar, R., Tsuji, N., Morales, T., Ozols, V. and Mehta, K. (1998). An ERp60-like protein from the filarial parasite *Dirofilaria immitis* has both transglutaminase and protein disulfide isomerase activity. Proc Natl Acad Sci U S A. 95, 531-6.

Chen, J.S., Sappington, T.W. and Raikhel, A.S. (1997). Extensive sequence conservation among insect, nematode, and vertebrate vitellogenins reveals ancient common ancestry. J Mol Evol 44, 440-51.

Claerebout, E., Smith, W.D., Pettit, D., Geldhof, P., Raes, S., Geurden, T. and Vercruysse, J. (2005). Protection studies with a globin-enriched protein fraction of *Ostertagia ostertagi*. Vet Parasitol 128, 299-307.

Cottee, P.A., Nisbet, A.J., ABS El-Osta, Y.G., Webster, T.L. and Gasser, R.B. (2006). Construction of gender-enriched cDNA archives for adult *Oesophagostomum dentatum* by suppressive-subtractive hybridization and a microarray analysis of expressed sequence tags. Parasitol 132, 691-708

Cox, G.N., Pratt, D., Hageman, R. and Boisvenue, R.J.. (1990). Molecular cloning and primary sequence of a cysteine protease expressed by *Haemonchus contortus* adult worms. Mol Biochem Parasitol 41, 25-34.

Craig, H., Wastling, J.M. and Knox, D.P. (2006). A preliminary proteomic survey of the in vitro excretory/secretory products of fourth–stage larval and adult *Teladorsagia circumcinta*. Parasitol 132, 535-543.

Cross, D.A. and Klesius, P.H. (1989). Soluble extracts from larval *Ostertagia ostertagi* modulating immune function. Int J Parasitol 19, 57-61.

Dalton, J.P., McGonigle, S., Rolph, T.P. and Andrews, S.J. (1996). Induction of protective immunity in cattle against infection with *Fasciola hepatica* by vaccination with cathepsin L proteinases and with hemoglobin. Infect Immun 64, 5066-5074.

Daub, J., Loukas, A., Pritchard, D.I. and Blaxter, M. (2000). A survey of genes expressed in adults of the human hookworm, *Necator americanus*. Parasitol 120, 171-84.

De Cock, H., Knox, D.P., Claerebout, E. and de Graaf, D.C. (1993). Partial characterization of proteolytic enzymes in different developmental stages of *Ostertagia ostertagi*. J Helminthol 67, 271-8.

de Graaf, D.C., Berghen, P., Moens, L., de Marez, T.M., Raes, S., Blaxter, M.L. and Vercruysse J. (1996). Isolation, characterization and immunolocalization of a globin-like antigen from *Ostertagia ostertagi* adults. Parasitol 113, 63-9.

De Maere, V., Vercauteren, I., Geldhof, P., Gevaert, K., Vercruysse, J. and Claerebout, E. (2005a). Molecular analysis of astacin-like metalloproteases of *Ostertagia ostertagi*. Parasitol 130, 89-98.

De Maere, V., Vercauteren, I., Gevaert, K., Vercruysse, J. and Claerebout, E. (2005b). An aspartyl protease inhibitor of *Ostertagia ostertagi*: molecular cloning, analysis of stage and tissue specific expression and vaccine trial. Mol Biochem Parasitol 14, 181-8.

De Marez, T., Cox, E., Vercruysse, J. and Goddeeris, B.M. (2000). Identification of *Ostertagia ostertagi* specific cells in bovine abomasal lymph nodes. Vet Immunol Immunopathol 73, 145-54.

Dzik, J.M. (2006). Molecules released by helminth parasites involved in host colonization. Acta Biochim Pol 53,33-64.

Eiler, H., Baber, W., Lyke, W.A. and Scholtens, R. (1981). Inhibition of gastric hydrochloric acid secretions in the rat given *Ostertagia ostertagi* (a gastric parasite of cattle) extract. Am J Vet Res 42, 498-502.

Elsasser, T.H., Sartin, J.L., McMahon, C., Romo, G., Fayer, R., Kahl, S. and Blagburn, B. (1998). Changes in somatotropic axis response and body composition during growth hormone administration in progressive cachectic parasitism. Domest Anim Endocrinol 15, 239-55.

Epe, C., Kohlmetz, C. and Schnieder, T. (1998). A recombinant protein disulfide isomerase homologue from *Ancylostoma caninum*. Parasitol Res 84, 763-6.

Fetterer, R.H., Hill, D.E. and Rhoads, M.L. (1999). Characterization of a hemoglobin-like protein from adult *Haemonchus contortus.* J Parasitol 85, 295-300.

Fox, M.T., Gerrelli, D., Pitt, S.R., Jacobs, D.E., Gill, M. and Gale, D.L. (1989a). *Ostertagia ostertagi* infection in the calf: effects of a trickle challenge on appetite, digestibility, rate of passage of digesta and liveweight gain. Res Vet Sci 47, 294-298.

Fox, M.T., Gerrelli, D., Pitt, S.R., Jacobs, D.E. and Simmonds, A.D. (1989b). *Ostertagia ostertagi* infection in the calf: effects of a trickle challenge on the hormonal control of digestive and metabolic function. Res Vet Sci 47, 299-304.

Fox, M.T. (1993). Pathophysiology of infection with *Ostertagia ostertagi* in cattle. Vet Parasitol 46,143-58.

Fox, M.T., Carroll, A.P., Hughes, S.A, Uche, U.E., Jacobs, D.E. and Vaillant, C. (1993). Gastrin and gastrin-related responses to infection with *Ostertagia ostertagi* in the calf. Res Vet Sci 54, 384-91.

Fox, M.T. (1997). Pathophysiology of infection with gastrointestinal nematodes in domestic ruminants: recent developments. Vet Parasitol 72, 285-308.

Fox, M.T., Uche, U.E., Vaillant, C., Ganabadi, S. and Calam, J. (2002). Effects of *Ostertagia ostertagi* and omeprazole treatment on feed intake and gastrin-related responses in the calf. Vet Parasitol 105, 285-301.

Fox, M.T., Reynolds, G.W., Scott, I., Simcock, D.C. and Simpson, H.V. (2006). Vagal and splanchnic afferent nerves are not essential for anorexia associated with abomasal parasitism in sheep. Vet Parasitol 135, 287-95.

Freedman, R.B., Hirst, T.R. and Tuite, M.F. (1994). Protein disulphide isomerase: building bridges in protein folding. Trends Biochem Sci 19, 331-6.

Frenkel, M.J., Dopheide, T.A., Wagland, B.M. and Ward, C.W. (1992). The isolation, characterization and cloning of a globin-like, host-protective antigen from the excretory-secretory products of *Trichostrongylus colubriformis*. Mol Biochem Parasitol 50, 27-36

Gamble, H.R. and Mansfield, L.S. (1996). Characterisation of excretory-secretory products from larval stages of *Haemonchus contortus.* Vet Parasitol 62, 291-305.

Gasbarre, L.C. (1997). Effects of gastrointestinal nematode infection on the ruminant immune system. Vet Parasitol 72, 327-337.

Gasbarre, L.C., Leighton, E.A. and Sonstegard, T. (2001). Role of the bovine immune system and genome in resistance to gastrointestinal nematodes. Vet Parasitol 98, 51-64.

Geldhof, P., Claerebout E., Knox, D.P., Agneessens J. and Vercruysse J. (2000). Proteinases released *in vitro* by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. Parasitol 121, 639-647.

Geldhof, P., Claerebout, E., Knox, D., Vercauteren, I., Looszova, A. and Vercruysse, J. (2002). Vaccination of calves against *Ostertagia ostertagi* with cysteine proteinase enriched protein fractions. Parasite Immunol 24, 263-270.

Geldhof, P., Vercauteren, I., Knox, D., De Maere, V., Van Zeveren, A., Vercruysse, J. and Claerebout E. (2003a). Protein disulphide isomerase of *Ostertagia ostertagi*: an excretory-secretory product of L4 and adult worms? Int J Parasitol 33, 129

Geldhof, P., Vercauteren, I., Gevaert, K., Staes, A., Knox, D.P., Vandekerckhove, J., Vercruysse, J. and Claerebout, E. (2003b). Activation-associated secreted proteins are the most abundant antigens in a host protective fraction from *Ostertagia ostertagi*. Mol Biochem Parasitol 128, 111-114.

Geldhof, P., Vercauteren, I., Vercruysse, J., Knox, D.P., van den Broeck, W. and Claerebout, E. (2004). Validation of the protective *Ostertagia ostertagi* ES-thiol antigens with different adjuvantia. Parasite Immunol 26, 37-43.

Gomez-Munoz, M.T., Canals-Caballero, A., Almeria, S., Pasquali, P., Zarlenga, D.S. and Gasbarre, L.C. (2004). Inhibition of bovine T lymphocyte responses by extracts of the stomach worm *Ostertagia ostertagi*. Vet Parasitol 120, 199-214.

Goud, G.N., Zhan, B., Ghosh, K., Loukas, A., Hawdon, J., Dobardzic, A., Deumic, V., Liu, S., Dobardzic, R., Zook, B.C., Jin, Q., Liu, Y., Hoffman, L., Chung-Debose, S., Patel, R., Mendez, S. and Hotez, PJ. (2004). Cloning, yeast expression, isolation, and vaccine testing of recombinant *Ancylostoma*-secreted protein (ASP)-1 and ASP-2 from *Ancylostoma ceylanicum*. J Infect Dis 189, 919-29.

Guilmeau, S., Buyse, M. and Bado, A. (2004). Gastric leptin: a new manager of gastrointestinal function. Curr Opin Pharmacol 4, 561-6.

Haag, E. (1995). The effect of *Haemonchus contortus* excretory/secretory products on abomasal secretion. Dr Med Vet Thesis, Hannover.

Hartman, D., Cottee, P.A., Savin, K.W., Bhave, M., Presidente, P.J., Fulton, L., Walkiewicz, M. and Newton, S.E. (2003). *Haemonchus contortus*: molecular characterisation of a small heat shock protein. Exp Parasitol. 104, 96-103.

Hauser, F., Roeben, C. and Hoffmann, W. (1992). xP2, a new member of the P-domain peptide family of potential growth factors, is synthesized in *Xenopus laevis* skin. J Biol Chem 267, 14451-14455

Hawdon, J.M., Jonest, B.F., Hoffman, D.R. and Hotez, P.J. (1996). Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. J Biol Chem 271, 6672-6678

Hawdon, J.M., Narasimhan, S. and Hotez, P.J. (1999). *Ancylostoma* secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum.* Mol Biochem Parasitol 99, 149-65.

Hertzberg, H. Lindström, E., Chen, D. and Håkanson, R. (1999). Excretory/secretory products of *Haemonchus contortus* suppress stimulation of parietal cells by inhibiting secretory activity of enterochromaffine-like (ECL) cells. Proceedings 17 th International Conference of the WAAVP, Copenhagen. Abstract a.202.

Hertzberg, H., Guscetti, F., Lischer, C., Kohler, L., Neiger, R. and Eckert J. (2000). Evidence for a parasite-mediated inhibition of abomasal acid secretion in sheep infected with *Ostertagia leptospicularis*. Vet J 159, 238-51.

Hoste, H. (2001). Adaptive physiological processes in the host during gastrointestinal parasitism. Int J Parasitol 31, 231-44.

Hotez, P.J., Narasimhan, S., Haggerty, J., Milstone, L., Bhopale, V., Schad, G.A. and Richards, F.F. (1992). Hyaluronidase from infective *Ancylostoma* hookworm larvae and

its possible function as a virulence factor in tissue invasion and in cutaneous larva migrans. Infect Immun 60, 1018-23.

Huber, A., Prosl, H., Joachim, A., Simpson, H.V. and Pedley, K.C. (2005). Effects of excretory/ secretory products of *Haemonchus contortus* on cell vacuolation. Parasitol Research 96, 290-295.

Jecock, R.M., Devaney, E. (1992). Expression of small heat shock proteins by the third-stage larva of *Brugia pahangi*. Mol Biochem Parasitol 56, 219-26.

Jennings, F.W., Armour, J., Lawson, D.D. and Roberts, R. (1966). Experimental *Ostertagia ostertagi* infections in calves: studies with abomasal cannulae. Am J Vet Res 27, 1249-1257.

Jordanova, R., Radoslavov, G., Fischer, P., Liebau, E., Walter, R.D., Bankov, I. and Boteva, R. (2005). Conformational and functional analysis of the lipid binding protein Ag-NPA-1 from the parasitic nematode *Ascaridia galli*. FEBS J 272, 180-9.

Karanu, F.N., Rurangirwa, F.R., McGuire, T.C. and Jasmer, D.P. (1993). *Haemonchus contortus*: identification of proteases with diverse characteristics 1362-371. Exp Parasitol 77, 362-71.

Kasahara, M., Gutknecht, J., Brew, K., Spurr, N., Goodfellow, P.N. (1989). Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. Genomics 5, 527-534.

Kennedy, M.W., Qureshi, F., Fraser, E.M., Haswell-Elkins, M.R., Elkins, D.B. and Smith, H.V. (1989). Antigenic relationships between the surface-exposed, secreted and somatic materials of the nematode parasites *Ascaris lumbricoides*, *Ascaris suum*, and *Toxocara canis*. Clin Exp Immunol 75, 493-500.

Kennedy, M.W., Brass, A., McCruden, A.B., Pricen, N.C., Kelly, S.M. and Cooper, A. (1995). The ABA-1 allergen of the parasitic nematode *Ascaris suum*: fatty acid and retinoid binding function and structural characterization. Biochemistry 34, 6700-10.

Kennedy, M.W. (2000). Introduction: lipid-binding proteins: novel aspects. Cell Mol Life Sci 57, 1343-4.

Khan, W.I. and Collins, S.M. (2004). Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. Parasite Immunol 26, 319-26.

Klesius, P.H., Washburn, S.M., Ciordia, H., Haynes, T.B. and Snider III, T.G. (1984). Lymphocyte reactivity to *Ostertagia ostertagi* L3 antigen in type I ostertagiasis. Am J Vet Res 45, 230-233.

Klesius, P.H., Haynes, T.B., Cross, D.A. and Ciordia, H. (1986). *Ostertagia ostertagi*: excretory secretory chemotactic substance from infective larvae as cause of eosinophil locomotion. Exp Parasitol 61, 120-5.

Klesius, P.H., Snider III, T.G., Horton, L.W. and Crowder, C.H. (1989). Visualization of eosinophil chemotactic factor in abomasal tissue of cattle by immunoperoxidase staining during *Ostertagia ostertagi* infection. Vet Parasitol 31, 49-56.

Klesius, P.H. (1993). Regulation of immunity to *Ostertagia ostertagi*. Vet. Parasitol 46, 63-79.

Knox, D. P., Smith, S.K. and Smith, W. D. (1999). Immunization with an affinity purified protein extract from the adult parasite protects lambs against infection with *Haemonchus contortus*. Parasite Immunol. 21, 201-210.

Knox, D.P. (2000). Development of vaccines against gastrointestinal nematodes. Parasitol 120, S43-S61.

Knox, D. P., Smith, S. K., Redmond, D. L. and Smith, W. D. (2005). Protection induced by vaccinating sheep with a thiol-binding extract of *Haemonchus contortus* membranes is associated with its protease components. Parasite Immunol. 27, 121-126.

Kokke, B.P., Leroux, M.R., Candido, E.P., Boelens, W.C. and de Jong W.W. (1998). *Caenorhabditis elegans* small heat-shock proteins Hsp12.2 and Hsp12.3 form tetramers and have no chaperone-like activity. FEBS Lett. 433, 228-32.

Krätzschmar, J., Haendler, B., Eberspaecher, U., Roosterman, D., Donner, P. and Schleuning, W.D. (1996). The human cysteine-rich secretory protein (CRISP) family. Primary structure and tissue distribution of CRISP-1, CRISP-2 and CRISP-3. Eur J of Bioch 236, 827-836.

Lawton, D.E., Wigger, H., Simcock, D.C. and Simpson, HV. (2002). Effect of *Ostertagia circumcincta* excretory/secretory products on gastrin release *in vitro*. Vet Parasitol 104, 243-55.

Lehmann, F.S., Golodner, E.H., Wang, J., Chen, M.C., Avedian, D., Calam, J., Walsh, J.H., Dubinett, S. and Soll, A.H. (1996). Mononuclear cells and cytokines stimulate gastric release from canine antral cells in primary culture. Am J Vet Res 270, G783-788.

Leroux, M.R., Batelier, B.J., Melki, R. and Candido, E.P. (1997a). Unique structural features of a novel class of small heat shock proteins. J Biol Chem. 272, 12847-12853.

Leroux, M.R., Melki, R., Gordon, B., Batelier, G. and Candido, E.P. (1997b). Structure-function studies on small heat shock protein oligomeric assembly and interaction with unfolded polypeptides. J Biol Chem. 272, 24646-56.

Li, B.W., Rush, A.C., Crosby, S.D., Warren, W.C., Williams, S.A., Mitreva, M. and Weil, G.J. (2005). Profiling of gender-regulated gene transcripts in the filarial nematode *Brugia malayi* by cDNA oligonucleotide array analysis. Mol Bioch Parasitol 143, 49-57.

Lillibridge, C.D., Rudin, W., Philipp, M.T. (1996). *Dirofilaria immitis*: ultrastructural localization, molecular characterization, and analysis of the expression of p27, a small heat shock protein homolog of nematodes. Exp Parasitol 83, 30-45.

Lippl, F., Kircher, F., Erdmann, J., Allescher, H.D. and Schusdziarra, V. (2004). Effect of GIP, GLP-1, insulin and gastrin on ghrelin release in the isolated rat stomach. Regul Pept 119, 93-8.

Lindquist, S. (1986). The heat-shock response. Annu Rev Biochem 55, 1151-91.

Loukas, A., Hintz, M., Linder, D., Mullin, N., Parkinson, J., Tetteh, K. and Maizels R. (2000). A Family of Secreted Mucins from the Parasitic Nematode *Toxocara canis* Bears Diverse Mucin Domains but Shares Similar Flanking Six-cysteine Repeat Motifs. J Biol Chem 275, 39600-39607.

MacLennan, K., Gallagher, M.P. and Knox, D.P. (1997). Stage-specific serine and metallo-proteinase release by adult and larval *Trichostrongylus vitrinus*. Int J Parasitol 27, 1031-6.

Martinez-Valladares, M., Godio-Fernandez, R., Vara-Del Rio, M.P., Martin, J.F. and Rojo-Vazquez, F.A. (2007). Expression of the recombinant protein disulphide isomerase of *Teladorsagia circumcincta*. Parasite Immunol. 29, 47-56.

McGibbon, A.M.n Christie, J.F., Kennedy, M.W. and Lee, T.D. (1990). Identification of the major *Ascaris* allergen and its purification to homogeneity by high-performance liquid chromatography. Mol Biochem Parasitol 39, 163-171.

McKellar, Q.A., Duncan, J.L., Armour, J. and McWilliam, P. (1986). Response to transplanted adult *Ostertagia ostertagi* in calves. Res Vet Sci 40, 367-371.

McKellar, Q.A., .Duncan, J.L., Armour, J., Lindsay, F.E.F. and McWilliam, P. (1987). Further studies on the response to transplanted adult *Ostertagia ostertagi* in calves. Res Vet Sci 42, 29-34.

McKellar, Q.A., Mostofa, M. and Eckersall, P.D. (1990a). *Ostertagia ostertagi* secretions and abomasal acid production. In: Proceedings of the Seventh International Congress of Parasitology, Paris, August 20-24, p. 831

McKellar, Q.A. (1993). Interactions of *Ostertagia* species with their bovine and ovine hosts. Int J Parasitol 23,451-462.

Merkelbach, P., Scott, I., Khalaf, S. and Simpson, H.V. (2002). Excretory/secretory products of *Haemonchus contortus* inhibit aminopyrine accumulation by rabbit gastric glands in vitro. Vet Parasitol 104, 217-228.

Meyvis, Y., Geldhof, P., Gevaert, K., Timmerman E., Vercruysse J. and Claerebout E. (2007). Vaccination against *Ostertagia ostertagi* with subfractions of the protective ES-thiol fraction. Vet Parasitol 149, 239-45.

Mizuki, N. and Kasahara M. (1992). Mouse submandibular glands express an androgen-related transcript encoding an acid epididymal glycoprotein-like molecule. Mol Cell Endocrinol 89, 25-32.

Mizuki, N., Sarapata, D.E., Garcia-Sanz, J.A. and Kasahara, M. (1992). The mouse male germ cell-specific gene TPX-1 - molecular-structure, mode of expression in spermatogenesis, and sequence similarity to 2 nonmammalian genes. Mammalian Genome 3, 274-280.

Moser, J.M., Freitas, T., Arasu, P. and Gibson, G. (2005). Gene expression profiles associated with the transition to parasitism in *Ancylostoma caninum* larvae. Mol Biochem Parasitol 143, 39-48.

Myllyharju, J., Kukkola, L., Winter, A.D. and Page, A.P. (2002). The exoskeleton collagens in *Caenorhabditis elegans* are modified by prolyl 4-hydroxylases with unique combinations of subunits. J Biol Chem 277, 29187–29196.

Murray, M., Jennings, F.W. and Armour, J. (1970). Bovine ostertagiasis: structure, function and mode of differentiation of the bovine gastric mucosa and kinetics of the worm loss. Res Vet Sci 11, 417-427.

Murray, J. and Smith, W.D. (1994). Ingestion of host immunoglobulin by three non-blood-feeding nematode parasites of ruminants. Res Vet Sci 57, 387-9.

Murray, J., Gregory, W.F., Gomez-Escobar, N., Atmadja, A.K. and Maizels, R.M. (2001). Expression and immune recognition of *Brugia malayi* VAL-1, a homologue of vespid venom allergens and *Ancylostoma* secreted proteins. Mol Biochem Parasitol 118, 89-96.

Nisbet, A.J. and Gasser, R.B. (2004). Profiling of gender-specific gene expression for *Trichostrongylus vitrinus* (Nematoda: Strongylida) by microarray analysis of expressed sequence tag libraries constructed by suppressive-subtractive hybridization. Int J Parasitol 34, 633-643.

Page, A.P. (1997). Cyclophilin and protein disulfide isomerase genes are co-transcribed in a functionally related manner in *Caenorhabditis elegans*. DNA Cell Biol 16, 1335-43.

Poole, C.B., Grandea, A.G., Maina, C.V., Jenkins, R.E., Selkirk, M.E. and McReynolds, L.A. (1992). Cloning of a cuticular antigen that contains multiple tandem repeats from the filarial parasite *Dirofilaria immitis*. Proc Natl Acad Sci USA 89, 5986-90.

Purewal, A., Fox, M.T., Shivalkar, P., Carroll, A.P., Uche, U.E., Vaillant, C. and Watkinson, A. (1997). Effects of *Ostertagia ostertagi* on gastrin gene expression and gastrin-related responses in the calf. J Physiol 498, 809-16.

Raghavan, N., Ghosh, I., Eisinger, W.S., Pastrana, D. and Scott, A.L. (1999). Developmentally regulated expression of a unique small heat shock protein in *Brugia malayi*. Mol Biochem Parasitol 104, 233-46.

Redmond, D. L. and Knox, D. P. (2004). Protection studies in sheep using affinity-purified and recombinant cysteine proteinases of adult *Haemonchus contortus*. Vaccine 22, 4252-4561.

Redmond, D.L., Smith, S.K., Halliday, A., Smith, W.D., Jackson, D.P., Knox, D.P. and Matthews, J.B. (2006). An immunogenic cathepsin F secreted by the parasitic stages of *Teladorsagia circumcincta.* Int J Parasitol 36, 277-286.

Rehman, A. and Jasmer, D.P. (1998). A tissue specific approach for analysis of membrane and secreted protein antigens from *Haemonchus contortus* gut and its application to diverse nematode species. Mol Biochem Parasitol 97, 55-68.

Reinke, V., Gil, I.S., Ward, S., Kazmer, K. (2004). Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. Development 131, 311-323.

Rhoads, M.L. and Fetterer, R.H. (1995). Developmentally regulated secretion of cathepsin L-like cytokine proteases by *Haemonchus contortus*. J Parasitol 81, 505-512.

Schallig, H.D.F.H., Van Leeuwen, M.A.W. and Cornelissen A.W.C.A. (1997a). Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep. Parasite Immunol 19, 447-453.

Schallig, H.D.F.H., Van Leeuwen, M.A.W., Verstrepen, B.E. and Cornelissen A.W.C.A. (1997b). Molecular characterization and expression of two putative protective excretory secretory proteins of *Haemonchus contortus*. Mol Bioch Parasitol 88, 203-213.

Scott, I., Stear, M.J., Irvine, J., Dick, A., Wallace, D.S. and McKellar, Q.A. (1998a). Changes in the zymogenic cell populations of the abomasa of sheep infected with *Haemonchus contortus*. Parasitol 116, 569-77.

Scott, I., Hodgkinson, S.M., Khalaf, S., Lawton, D.E.B., Collett, M.G., Reynolds, G.W., Pomray, W.E. and Simpson, H.V. (1998b). Infection of sheep with adult and larval *Ostertagia circumcinta*: abomasal morphology. Int J Parasitol 28, 1383-1392.

Scott, I. and McKellar, Q.A. (1998). The effects of excretions/secretions of *Ostertagia circumcincta* on ovine abomasal tissues *in vitro*. Int J Parasitol 28, 451-60.

Scott, I., Dick, A., Irvine, J., Stear, M.J. and McKellar, Q.A. (1999). The distribution of pepsinogen within the abomasa of cattle and sheep infected with *Ostertagia* spp. and sheep infected with *Haemonchus contortus.* Vet Parasitol 82, 145-59.

Scott, I., Khalaf, S., Lawton, Simcock, D.C., Knight, C.G., Reynolds, G.W., Pomroy, W.E. and Simpson, H.V. (2000). A sequential study of the pathology associated with the infection of sheep with adult and larval *Ostertagia circumcinta.* Vet Parasitol 89, 79-94.

Selkirk, M.E., Gregory, W.F., Jenkins, R.E. and Maizels, R.M. (1993). Localization, turnover and conservation of gp15/400 in different stages of *Brugia malayi*. Parasitol 107, 449-57.

Serrano, M.T., Lanas, A.I., Lorente, S. and Sainz, R. (1997). Cytokine effects on pepsinogen secretion from human peptic cells. Gut 40, 42-8.

Simpson, H.V., Lawton, D.E.B., Simcock, D.C., Reynolds, G.W. and Pomroy, W.E. (1997). Effects of adult and larval *Haemonchus contortus* on abomasal secretion. Int J Parasitol 27,825-831.

Simpson, H.V., Simpson, B.H., Simcock, D.C., Reynolds, G.W. and Pomroy, W.E. (1999). Abomasal secretion in sheep receiving adult *Ostertagia circumcincta* that are prevented from contact with the mucosa. NZ Vet J 47, 20-24.

Snider III, T.G., Willliams, J.C., Karns, P.A., Romaire, T.L., Trammel, H.F. and Kearney, M.T. (1986). Immunosuppression of lymphocyte blastogenesis in cattle infected with *Ostertagia ostertagi* and/or *Trichostrongylus axei*. Vet Immunol Immunopathol 11, 251-64.

Solovyova, A.S., Meenan, N., McDermott, L., Garofalo, A., Bradley, J.E., Kennedy, M.W. and Byron, O. (2003). The polyprotein and FAR lipid binding proteins of nematodes: shape and monomer/dimer states in ligand-free and bound forms. Eur Biophys J. 32, 465-76.

Strayer A, Wu Z, Christen Y, Link CD, Luo Y. (2003). Expression of the small heat-shock protein Hsp16-2 in *Caenorhabditis elegans* is suppressed by *Ginkgo biloba* extract EGb 761. FASEB J. 17, 2305-7.

Stringfellow, F. and Madden, P.A. (1979). Effects of *Ostertagia ostertagi* on pepsinogen granules of chief cells from the abomasal mucosa correlated with selected plasma and abomasal proteins. Proc Helminthol Soc Wash 46, 223-239.

Tanovic, A., Fernandez, E. and Jimenez, M. (2006). Alterations in intestinal contractility during inflammation are caused by both smooth muscle damage and specific receptor-mediated mechanisms. Croat Med J 47, 318-26.

Tawe, W., Pearlman, E., Unnasch, T.R. and Lustigman, S. (2000). Angiogenic activity of *Onchocerca volvulus* recombinant proteins similar to vespid venom antigen 5. Mol Biochem Parasitol 109, 91-9.

Thompson, F.J., Martin, S.A. and Devaney, E. (1996). *Brugia pahangi*: characterisation of a small heat shock protein cDNA clone. Exp Parasitol 83, 259-66.

Tort, J., Brindley, P.J., Knox, D.P., Wolfe, K.H. and Dalton, J.P. (1999). Proteinases and associated genes of parasitic helminths. Adv In Parasitol 43, 161-266.

Tweedie , S., Grigg, M.E., Ingram, L. and Selkirk, M.E. (1993). The expression of a small heat shock protein homologue is developmentally regulated in *Nippostrongylus brasiliensis*. Mol Biochem Parasitol 61, 149-53.

Venkova, K. and Greenwood-van Meerveld, B. (2006). Long-lasting changes in small intestinal transport following the recovery from *Trichinella spiralis* infection. Neurogastroenterol Motil 18, 234-42.

Vercauteren, I., Geldhof, P., Peelaers, I., Claerebout, E., Berx, G. and Vercruysse, J. (2003). Identification of excretory-secretory products of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. Mol Biochem Parasitol 126, 201-208.

Vercauteren, I., Geldhof, P., Peelaers, I., Van den Broeck, W., Gevaert, K. and Claerebout, E. (2004). Vaccination with an *Ostertagia* polyprotein allergen protects calves against a homologous challenge infection. Inf Imm 72, 2995-3001.

Vercauteren, I., De Maere, V., Vercruysse, J., Stevens, M., Gevaert, K. and Claerebout, E. (2006). A small heat shock protein of *Ostertagia ostertagi*: stage-specific expression, heat inducibility, and protection trial. J Parasitol 92, 1244-50.

Vervelde, L., Van Leeuwen, M.A., Kruidenier, M., Kooyman, F.N., Huntley, J.F., Van Die, I. and Cornelissen, A.W. (2002). Protection studies with recombinant excretory/secretory proteins of *Haemonchus contortus*. Parasite Immunol 24, 189-201.

Visser, A., Van Zeveren, A.M., Meyvis, Y., Peelaers, I.,Van den Broeck, W., Gevaert, K., Vercruysse, J., Claerebout, E. and Geldhof, P., 2007. Gender enriched transcription of activation associated secreted proteins (ASPs) in *Ostertagia ostertagi*. Int J Parasitol, in press.

Washburn, SM and Klesius, P.H. (1984). Leukokinesis in bovine ostertagiasis: stimulation of leukocyte migration by *Ostertagia*. Am J Vet Res 45, 1095-1098.

Wijffels, G. L., Salvatore, L., Dosen, M., Waddington, J., Wilson, L., Thompson, C., Campbell, N., Sexton, J., Wicker, J., Bowen, F., Friedel, T. and Spithill, T. W. (1994). Vaccination of sheep with purified cysteine proteinases of *Fasciola hepatica* decreases worm fecundity. Exp Parasitol 78, 132-148.

Wildblood, L.A., Kerr, K., Clark, D.A., Cameron, A., Turner, D.G. and Jones, D.G. (2005). Production of eosinophil chemoattractant activity by ovine gastrointestinal nematodes. Vet Immunol Immunopathol 107, 57-65.

Williamson, A.L., Lustigman, S., Oksov, Y., Deumic, V., Plieskatt, J., Mendez, S., Zhan, B., Bottazzi, M.E., Hotez, P.J. and Loukas, A. (2006). *Ancylostoma caninum* MTP-1, an astacin-like metalloprotease secreted by infective hookworm larvae, is involved in tissue migration. Infect Immun 74, 961-7.

Winter, A.D., McCormack, G. and Page, A.P. (2007). Protein disulphide isomerase activity is essential for viability and extracellular matrix formation in the nematode *Caenorhabditis elegans*. Dev Biol 308, 449-461.

Wisse, B.E., Ogimoto, K., Morton, G.J., Wilkinson, C.W., Frayo, R.S., Cummings, D.E. and Schwartz, M.W. (2004). Physiological regulation of hypothalamic IL-1beta gene expression by leptin and glucocorticoids: implications for energy homeostasis. Am J Physiol Endocrinol Metab 287, 107-13.

Yang, C., Gibbs, H.C. and Xiao, L. (1993). Immunologic changes in *Ostertagia ostertagi*-infected calves treated strategically with an anthelmintic. Am J Vet Res 54, 1074-83.

Yasunaga, Y., Shinomura, Y., Kanayama, S., Higashimoto, Y., Yabu, M., Miyazaki, Y., Murayama, Y., Nishibayashi, H., Kitamura, S. and Matsuzawa, Y. (1997). Mucosal interleukin-1 beta production and acid secretion in enlarged fold gastritis. Aliment Pharmacol Ther 11, 801-9.

Yatsuda, A.P., Eysker, M., Vieira-Bresan, M.C.R. and De Vries, E. (2002). A family of activation associated secreted protein (ASP) homologues of *Cooperia punctata*. Res Vet Sci 73, 297-306.

Yatsuda, A. P., Krijgsveld, J., Cornelissen, A. W., Heck, A. J. and de Vries, E. (2003). Comprehensive analysis of the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition. J Biol Chem 278, 16941-16951.

Yatsuda, A., Bakker, N., Krijgsveld, J., Knox, D.P., Heck, A.J.R. and de Vries, E. (2006). Identification of secreted cysteine proteases from the parasitic nematode *Haemonchus contortus* detected by biotinylated inhibitors. Inf Imm 74, 1989-1993.

Young, C.J., MvKeand, J.B. and Knox, D.P. (1995). Proteinases released *in vitro* by the parasitic stages of *Teladorsagia circumcincta*, an ovine abomasal parasite. Parasitol Today 110, 465-471.

Objectives

1. Objectives of the study

As discussed in chapter 1, the abomasal parasite, *Ostertagia ostertagi*, drastically modulates its environment in the host. The mechanisms used by the parasite to change the abomasal environment are largely unknown, but an important role for parasite excretory-secretory products (ESPs) has been implicated. Furthermore, identification of interacting ESPs could open new perspectives for vaccination research and drug development. Therefore, the overall objective of this thesis was to study *Ostertagia ostertagi* ESPs and their possible interaction with bovine abomasal proteins. First, we assessed the use of phage display for the discovery and evaluation of the interactions of ESPs with the host (Chapter 2). Secondly, we proceeded with the analysis of novel and nematode-specific ESPs, more specifically:

1. A novel secreted protein family for selected members of the subfamily Ostertagiinae (chapter 3)
2. Transthyretin-like proteins (chapter 4)

Chapter 2

Assessment of the cDNA phage display technology to study the interaction between the gastrointestinal parasite *Ostertagia ostertagi* and its bovine host

1. Introduction

Since its original design by Smith (1985), multiple studies have described the filamentous phage display system as a useful screening method for the isolation of interacting ligands. Foreign DNA sequences have been expressed as fusions with the coat proteins on the phage surface, thereby linking phenotype to genotype. The phages expressing the desired ligand can be isolated from the large pool of recombinant phages by an affinity selection protocol, also called biopanning. By this means, a variety of peptides, proteins and antibody chains but even cDNA expression libraries have been displayed on phage particles. Numerous examples that demonstrate the potential of this method can be found in the human clinical research, for example the isolation of tumor specific antibodies, the identification of cancer vaccine candidates or the isolation of specific ligands out of a random peptide library (reviewed by Azzazy and Highsmith, 2002).

The phage display technology might also be useful in parasitological research. As discussed in chapter 1, helminth parasites drastically modulate their environment. Changes in stomach pH rapidly occur during an abomasal nematode infection in ruminants and result in a reduction of pepsinogen activation, increased gastrin secretion and reduced bacteriostatic effect (Simpson, 2000). The abomasal morphology is also drastically modified by developing abomasal nematodes: mucous cell hyperplasia, epithelial cell damage, nodular appearance of parasitized glands and accumulation of inflammatory cells such as eosinophils, neutrophils and lymphocytes (Scott *et al*., 1998). Although the mechanisms used by the parasites to change the abomasal environment are largely unknown, an important role has been attributed to parasite excretory-secretory products (ESPs). As described in chapter 1, several authors have implicated the participation of parasite ESPs in elevated stomach pH (Simpson *et al*., 1999; Eiler *et al*., 1981), pepsinogen secretion (McKellar *et al*., 1990a) and eosinophil recruitment (Wildblood *et al*., 2005). However, the nature of these secreted modulating factors and their ligands is unknown. In this study we have assessed the use of the filamentous M13 pVI phage display system to identify such molecules and their ligands. Two approaches were used during this assessment. In the first approach (Fig 2.1, exp. 1) native ESPs from both the L4 and adult life stage were screened against a phage display cDNA library produced from abomasal cells. Subsequently, a second experiment was set up, in which native membrane proteins of the abomasal epithelial cells were screened against an *O. ostertagi* phage display library.

Interaction

O.ostertagi	⟷	**Host (abomasum)**
Exp. 1 Native L4 and adult *O. ostertagia* ESPs	⟷	Abomasal phage display library
Exp. 2 *O. ostertagi* phage display library	⟷	Native membrane proteins of abomasal epithelial cells

Figure 2.1 Experimental design

In the following paragraphs, a short introduction on phage display and its components will be given.

1.1. Filamentous phage

Bacteriophages are viruses which infect bacteria. The most widely used ones infect *Escherichia coli.* In phage display, most work has been done on the filamentous phage (M13, fd, f1) but also more recently developed bacteriophage lambda vectors have been successful. The M13 phage has a long (~900 nm) and narrow (~7 nm) protein coat that encases a circular ssDNA molecule. The genome (6407 nucleotides) encodes for 11 proteins, five of which are exposed on the capsid and six of which are involved in phage maturation (DNA replication and phage assembly) inside the *E. coli* host (Fig 2.2). The filamentous phage will only infect *E. coli* cells carrying the F plasmid since the phage must adsorb to the F pilus to gain entry to the cells.

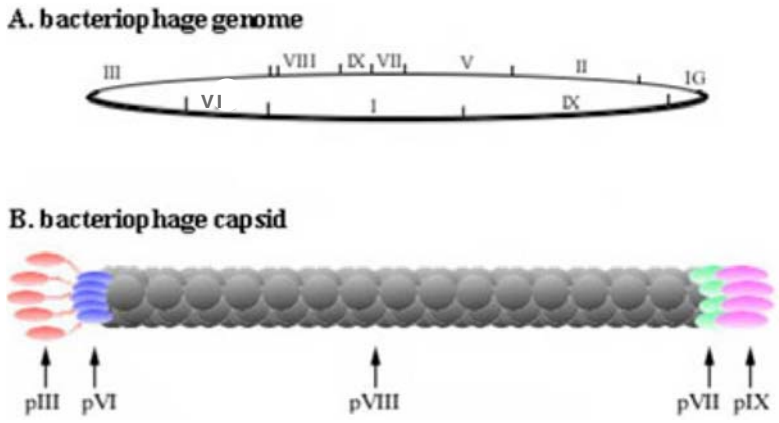


Figure 2.2 Phage structure: genome and capsid. (IG= intergenic region) http://www.helsinki.fi/bioscience/biochemistry/koivunen.htm

The phage coat is largely comprised of 2700 copies of major coat protein pVIII (or p8) together with four minor capsid proteins, pIII, pVI, pVII and pIX which are located at the ends of the filamentous particle. It should be noted that the coat's dimensions are flexible and the number of p8 copies adjusts to accommodate the size of the single stranded genome it packages. At one end of

the phage particle, there are five copies of the surface exposed pIX (p9) and a more buried pVII protein (p7). The other end is composed of five copies of the surface exposed pIII (p3) and the less exposed accessory protein, pVI (p6). They are the first proteins to interact with the *E. coli* host during infection (Fig 2.2) (McCafferty and Johnson, 1996).

1.2. Phage display

In the beginning of phage display, foreign polypeptides were fused to the amino-terminus of coat proteins pIII or pVIII. The DNA that encodes this polypeptide is housed within the virion. However large polypeptides hinder the coat protein function and assembly and may not be incorporated in the phage particle. To overcome these limitations, a phagemid system was developed. Foreign DNA sequences are fused to an additional coat protein gene that is encoded by a phagemid vector. Superinfection of the host cells with helper phages delivers the necessary wild type proteins required for the phage particle formation. These hybrid particles are composed of a phagemid DNA encased by mainly wild type coat proteins and a few fusion proteins (Fig 2.3).

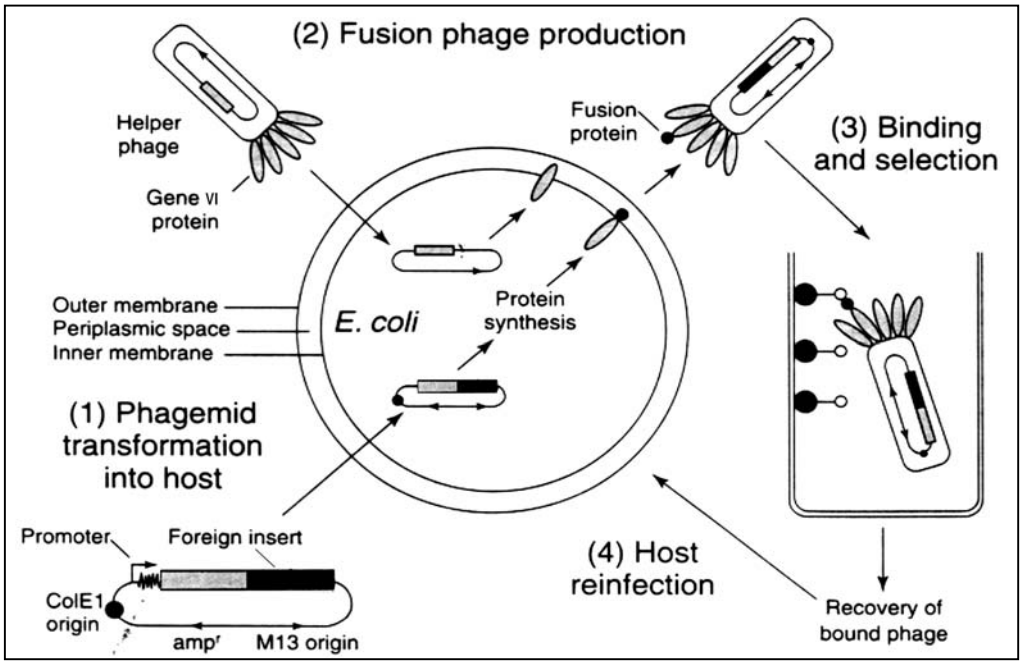


Figure 2.3 Production of phagemid particles. Adapted from Jefferies (1998)

The proteins displayed range from short amino acid sequences to antibody fragments, enzymes, cDNA and hormones. There are several display formats depending on the utilised coat protein and the manner of fusion (N- or C-terminal). Short proteins or peptides are mainly displayed as N-terminal fusions to the pIII or pVIII protein (Smith and Scott, 1993). Also cDNA expression libraries have been expressed on the phage surface: this appeared to be a challenging task because direct fusions of cDNA to the N-terminus of phage coat proteins pIII or pVIII are not suitable due to translational stop codons present in the 3' end of the cDNA. These limitations were overcome with the pVI C terminal cloning system (Jespers *et al*., 1995; Fransen *et al*., 1999; Viaene *et al*., 2001). Jespers *et al*. (1995) designed three phagemid vectors for functional expression of cDNA libraries on the surface of filamentous phage by fusion to the C-terminus of the minor phage coat protein pVI. Each phagemid vector (pG6A, pG6B and pG6C) represents one reading frame assuring correct translation of each protein (Viaene *et al.,* 2001). A second method was developed by Crameri and Suter (1993). Their approach was based on the leucine zipper interaction between Fos and Jun proteins that dimerize to form an oncogenic transcription factor Activator Protein-1. The pJuFo phagemid vector was constructed to link the C-terminus of pIII to the Jun gene while the cDNA products were fused to the C-terminus of the Fos gene. In the periplasm heterodimerisation occurs between the Fos-pIII and Jun-cDNA fusion proteins. Recently also C-terminal fusions of polypeptides to pIII and pVIII coat proteins have been reported (Fuh *et al*., 2000; Fuh and Sidhu, 2000). Obviously, here we have discussed only a fraction of different systems available nowadays. The development of novel innovative phagemid display systems and improvement of the current phagemid systems is an ongoing process.

Phages displaying a protein that specifically binds to a target protein can be enriched by biopanning (Fig 2.4). Briefly, the target of interest is immobilized on a plate and incubated with the phage display library. Phages displaying a complementary protein to the target are allowed to bind. Non-binding phages are then washed away while binding phages are eluted. Infection of bacteria with the binding phage results in phage amplification. Successive rounds of biopanning enrich the pool of phages, with clones that specifically bind the target. DNA sequencing of the phagemid insert determines the amino acid sequence of the binding proteins/peptides. However, phage selection is not limited to solid support biopanning as described above but has also been used *in vivo* and against intact cells for selection of tissue and cell targeting proteins (Arap, 2005).

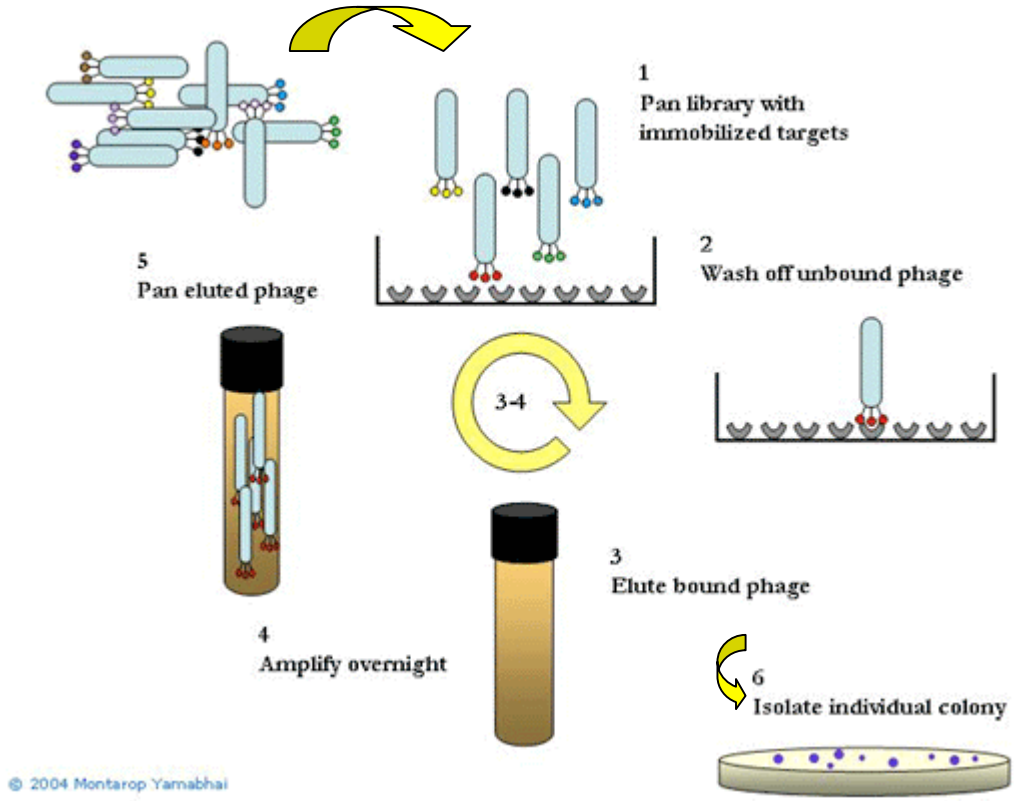


Figure 2.4 Biopanning (http://www.sut.ac.th/iat/biotech/PhDworkshop/)

2. Materials and Methods

2.1. First approach: parasite ES products versus abomasal phage library

2.1.1. Isolation of biological material

Two parasite naïve calves were infected with 300,000 and 100,000 infective L3 *O. ostertagi* larvae, respectively. Upon necropsy, L4 and adult *O. ostertagi* infected abomasal tissues were collected at the fundic region 10 and 21 days after infection, respectively. The abomasal flaps were washed in 1 x PBS and frozen by plunging into liquid nitrogen.

L4, adult *O. ostertagi* and their corresponding ES products were also collected upon slaughter as described in Geldhof *et al.* (2000). L4 and adults were harvested by opening the abomasum and placing it on a 220µm mesh sieve in a 0.9 % NaCl solution containing penicillin and streptomycin at 37°C for 5 hours. The collected parasites were rinsed thoroughly with the 0.9 % NaCl/penicillin/streptomycin solution and maintained for 48 hours at 37°C and 5% CO2 in sterile RPMI 1640 medium supplemented with gentamycin, penicillin, streptomycin, L-glutamin and amphotericine B. Parasite viability was checked at 24 and 48 hours based on structural integrity and motility. After the incubation period, the culture supernatants were filtered, concentrated and dialysed against PBS at 4°C. This recovered material can now be referred to as ESP.

2.1.2. RNA extraction

Total RNA was extracted by grinding the frozen abomasal tissue samples in a mortar in liquid nitrogen followed by TRIZOL-treatment (GibcoBRL, Life technologies). The RNA pellet was subsequently treated with DNAse I, air dried and dissolved in an appropriate volume of DEPC-treated water. The quality of the extracted total RNA and mRNA was verified on an agarose gel and their concentration was measured spectrophotometrically. Approximately 500 ng mRNA (isolated with mRNA Separator Kit, Clontech) was transcribed to double stranded cDNA with the SMART™cDNA Library Construction Kit (BD Biosciences). The cDNA was digested with Sfi I restriction enzyme (Promega).

2.1.3. Construction of abomasal phage display libraries

A double stranded linker with the required SfiI restriction sites (for unidirectional cloning) was constructed by annealing 2 complementary primers (table 2.1) via gradual cooling from 94°C to room temperature. This linker was cloned into the pG6A, pG6B and pG6C phagemid vectors (kindly provided by Dr. L. Jespers), followed by digestion with the SfiI restriction enzyme (Promega). The abomasal cDNAs were unidirectionally fused to the 3' end of the M13 gene VI expressed by pG6A, pG6B and pG6C phagemid vectors (each representing one of the three reading frames). The ligation mixtures were electroporated into

Top10F' or Omnimax™2 T1 Phage-Resistant *E. coli* cells (Invitrogen) and plated out on Luria Bertani agar plates supplemented with 100µg/ml ampicilline and 20µg/µl tetracycline (LBAT). After incubation overnight (37°C), colonies were scraped into liquid LBAT medium. The optical density OD_{600} of the cell mixture was measured with the spectrophotometer and (if needed) diluted to 40 OD_{600} units per ml with liquid LBAT medium. Glycerol stocks were prepared by adding sterile glycerol to the concentrated cell stock and snapfreezing the mixtures. The final cell concentration is 20 OD_{600} units per ml, which corresponds to approximately 1.6×10^{10} cells per ml.

Table 2.1 Sfi linker for pG6 A, B, C

Name	DNA sequence (5' to 3')
pGA+C Sfilinker5'	CACTAGGCCATTACGGCCTGCAGGATCCGGCCGCCTCGGCCTAACAGT
pGA+C Sfilinker3'	GTTAGGCCGAGGCGGCCGGATCCTGCAGGCCGTAATGGCCTAGTGACT
pGB Sfilinker5'	CACAAGGCCATTACGGCCTGCAGGATCCGGCCGCCTCGGCCTAACAGT
pGB Sfilinker3'	GTTAGGCC GAGGCGGCCGGATCCTGCAGGCCGTAATGGCCTTGTGACT

2.1.4. Phagemid rescue

Approximately 8×10^8 cells (50 µl of the glycerol stock) were inoculated into 20 ml of LBAT and grown at 37°C with shaking (250 rpm) until the culture reached an OD_{600} = 0.5-0.6. Phagemid particles were rescued from the pG6-cDNA libraries by superinfection of the cultures with 10^{11} R408 helper phages (Promega) or VCSM13 helper phages (Stratagene). The culture was incubated for 30 min at 37°C without shaking followed by 30 min at 37°C with slow shaking (150-180 rpm). The growth medium was then changed by centrifugation for 10 min at 1500 x g. The infected cells were resuspended in 100 ml of prewarmed LBAT and grown overnight with shaking (250 rpm) at 30°C. The next day, the phage supernatant was collected by transferring the overnight cultures in 50 ml polypropylene tubes and centrifuging them at 3500 x g for 30 min at 4°C. The phages were precipitated after incubation for 1 h on ice with 1/5 volume of 20% polyethyleenglycol6000/2.5M NaCl. The phages were centrifuged down at 3500 x g for 20 min at 4°C. The supernatant was discarded and the phage pellet resuspended in 1 ml of PBS. Remaining cell debris was removed by an additional centrifugation at 14000 x g in a microcentrifuge. Finally, the phage titre was measured at 260 nm. One OD260 nm corresponds to 44.28×10^{10} phages per ml.

2.1.5. Biopanning

The panning procedure was done as described earlier by McCafferty and Johnson (1996). Briefly, L4 or Adult *O. ostertagi* ES products (25 µg/2ml 1 X

PBS) were coated overnight on a Nunc Maxisorp immunotube followed by 3 wash steps with 1 X PBS and a blocking step with 2% Skim Milk (in 1X PBS, Gloria) for 1 or 2 hours at room temperature. Approximately 10^{12} phagemid particles (in 2% Skim milk, 1 X PBS) were incubated with the immobilised targets for 2 hours. After several wash steps with PBS and PBST, the bound phages were eluted from the immunotube with 1 ml 100mM Triethylamine (freshly prepared, pH 11) for 5 min on an end-over end rotor. The eluted phages were neutralized immediately with 0.5 ml 1M Tris-HCl pH 7.4. Log phase Top10 F' or Omnimax™2 T1 Phage-Resistant *E. coli* cells were infected with the eluted phages and plated out on LBAT agar plates. A small sample of the infected *E. coli* was diluted and also plated out to determine the titre of the eluted phages. Enriched phage libraries were generated by helper phage superinfection as described above. These phagemid particles were used in a next panning round. This procedure was repeated 3 to 4 times depending on the enrichment factor (output phages/ input phages). After the final panning round, the infected *E. coli* were plated out in such a manner that the individual colonies could be isolated for PCR-colony screening with pG6 specific primers (pG6F: 5'-TTGGATTGGGATAAAGGTGGAGGC-3'; pG6R: 5'-CGGTTGTAAAACGGCCAGT-3'). The DNA sequence data were assembled and submitted to nucleic acid and protein database (http://www.ncbi.nlm.nih.gov/BLAST/, http://compbio.dfci.harvard.edu/tgi/) searches.

2.1.6. Monoclonal phage production and dot blot

In a V-shaped 96-well plate (Nunc) with lid, overnight mini-cultures of individual colonies were grown in 100 µl LBAT at 37°C with shaking (150-180 rpm). The following day, 5 µl of the mini-culture was inoculated in 150 µl fresh LBAT medium and grown for 1 hour 15 min at 37°C with shaking. Again, phagemid particles were rescued by addition of helper phages as described above. The cultures were grown overnight at 30°C with shaking (150-180 rpm). The next day the phage supernatant was collected after centrifugation for 10 min at 2800 rpm in a swinging bucket. Approximately 25-50 µl of the phage supernatant was used in the monoclonal dot blot protocol.

Approximately 2 to 3 µg of adult and L4 *O. ostertagi* ESPs were spotted on a nitrocellulose membrane. Soybean trypsin inhibitor, a random plant protein, was spotted in the same amount as a negative control. Non-specific binding was blocked with 10 % horse serum (HS) in 1 x PBS containing 0.05 % Tween 20 (Sigma) (PBST). Subsequently, the membrane was incubated for 2 hours with the phage mixture (50 µl phage supernatant in 1ml 1x PBST + 2% HS). Then, the membrane was washed extensively with PBST (3 x 5 min) and incubated for 1 hour (with shaking) with mouse anti-M13 pVIII antibodies (GE healthcare, 1:2000 in PBST with 2% HS). This was followed by another series of wash steps. Finally, the membrane was incubated for 1 hour (with shaking) with affinity purified rabbit anti-mouse IgG (Sigma, 1:5000 in PBST with 2 % HS) conjugated

with horse-radish peroxidase. 3.3 Diaminobenzidine tetrachloride in PBS and 0.02 % H_2O_2 were added to detect bands.

2.2. Second approach: abomasal membrane proteins versus *O. ostertagi* phage library

2.2.1. Construction of adult *O. ostertagi* phage display library and phagemid rescue

The same protocol, as described in 2.1.3, was followed for construction of an *Ostertagia* phage display library. Briefly, total RNA was extracted by grinding the frozen adult *O. ostertagi* in a mortar in liquid nitrogen and was transcribed to double stranded cDNA. The *Ostertagia* cDNAs were cloned into the pG6A, pG6B and pG6C phagemid vectors and transformed into *E. coli* cells. Phagemid rescues of the *O. ostertagi* phage display libraries were done as described earlier (2.1.4).

2.2.2. Isolation of abomasal cells and cell membrane fraction

Epithelial cells of the abomasal fundic region were isolated as described in Almeria *et al*. (1997). Small tissue sections were taken from the abomasal folds and washed with Hanks' balanced salt solution (HBSS) without calcium and magnesium (Invitrogen). The residual fat was removed and the tissues were stored on ice. Subsequently, the tissues were incubated twice (30 – 60 min) at 37°C (200-250 rpm) in 100 ml HBSS medium supplemented with 1 mM DTT (removal of mucus) and 1.5 mM EDTA. The cell supernatants were filtered through organza to remove the mucus. The cell suspension was centrifugated for 10 min at 4°C and 1811 x g and the cell pellet was resuspended in HBSS medium. This wash step was repeated 3 times. Finally, the cells were coloured with negrosin for counting and checking their viability with light microscopy.

Approximately 5 x 10^6 cells were processed with the Cell Compartiment kit (Qiagen, Benelux BV.), designed for subcellular fractionation of intact cells, resulting in selective isolation of the membrane fraction of the abomasal epithelial cells.

2.2.3. Biopanning

The biopanning procedure started with coating the immunotube with approximately 20 µg (in PBS) of the membrane fraction of abomasal cells. In total, 4 panning rounds were performed with the *Ostertagia* phage display libraries.

A similar biopanning protocol was followed with intact abomasal cells, used as target molecules. 10^6 cells were maintained at 37°C and 5% CO2 in 5 ml sterile HBSS medium during a 1-2 hour incubation step with 10^{12} phagemid particles. This was followed by 3 extensive washing steps with PBST on an end-over-end rotor at 4 °C. Elution, neutralisation and reinfection of the binding phages were done as described above (2.1.4 and 2.1.5).

2.2.4. Western blotting

Three samples of approximately 4 µg of the membrane fraction were separated on NuPAGE® 10 % Bis-Tris Gels (Invitrogen) under reducing conditions and subsequently blotted on to a PVDF membrane. Non-specific binding was blocked with 10 % HS (in PBST). The 3 blots were incubated with 10^{12} pG6A, B, C phagemid particles (in 2 ml PBS + 2% HS) respectively, at 37 °C for 4 hours. Subsequently, the blots were washed extensively with PBST (overnight at 4 °C). The following day, they were incubated for 1 hour (with shaking) with mouse anti-M13 pVIII antibodies (GE healthcare, 1:2000 in PBST with 2% HS). This was followed by another series of wash steps. Finally, they were incubated with affinity purified rabbit anti-mouse IgG (Sigma, 1:5000 in PBST with 2 % HS) conjugated with horse-radish peroxidase. After washing with PBST, 3.3 diaminobenzidine tetrachloride in PBS and 0.02 % H_2O_2 were added to detect bands.

Adult *O. ostertagi* ESPs were biotinylated according to the manufacturer's protocol (Roche). Approximately 4 µg of the membrane fraction was separated on NuPAGE® 10 % Bis-Tris Gels (Invitrogen) under reducing conditions and subsequently blotted on to a PVDF membrane. Non-specific binding was blocked with 10 % HS (in PBST). The blotted membrane fraction was probed with 10 µg biotine labelled adult *O. ostertagi* ESPs for 4 hours with shaking at room temperature. Subsequently, the blot was washed with PBST and incubated with streptavidin (Sigma, 1:5000 in PBST with 2 % HS) conjugated with horse-radish peroxidase for 1 hour with shaking. After washing with PBST, 3.3 diaminobenzidine tetrachloride in PBS and 0.02 % H_2O_2 were added to detect bands. Several controls were also included on the blot: negative controls for endogenous biotinylation of the membrane fraction (4 µg) and adult ESPs (10 µg) but also a positive control to check the biotine labelling of adult ESPs (10 µg).

3. Results and discussion

Three abomasal (infected with adult *O. ostertagi*) cDNA phage display libraries were obtained: 1.26 x 10^5 clones in pG6A, 7.1 x 10^6 clones in pG6B and 9 x 10^6 clones in pG6C. Approximately 20 individual clones were selected at random and their insert sizes were determined by PCR screening with pG6 specific primers followed by sequencing. Inserts ranging from 300 to 1600 bp were found in 80% of the clones (Fig 2.5) with an average size of approximately 600 bp. Similar results were obtained for the L4 infected abomasal cDNA libraries. A sufficient number of clones was obtained in the 3 phagemid vectors (1.21 x 10^6 clones in pG6A, 1.4 x 10^5 clones in pG6B and 1,09 x 10^6 clones in pG6C) (data not shown).

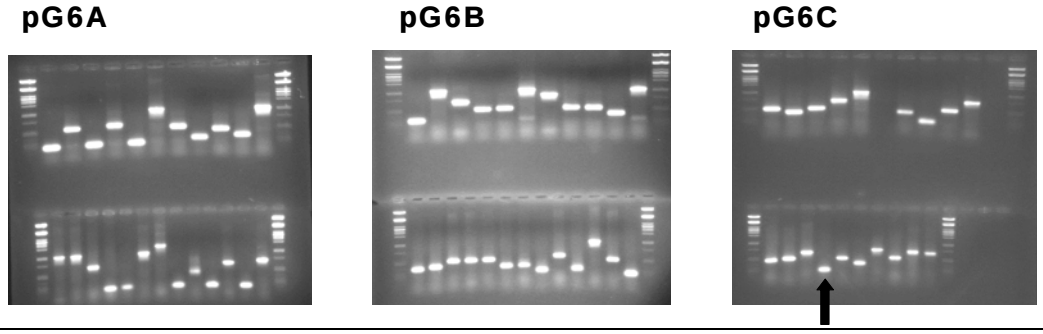


Figure 2.5 Abomasal cDNA phage display libraries in pG6A, B and C phagemid vectors of abomasum infected with adult *O. ostertagi*. Clones without a cDNA insert result in a PCR fragment of approximately 200 bp. One example is indicated with an arrow.

Three to four rounds of biopanning resulted in a gradual enrichment of the interacting phagemid particles. This is demonstrated in figure 2.6. Simultaneously, it was observed by PCR analysis that the number of different insert sizes decreased after each panning round (data not shown).

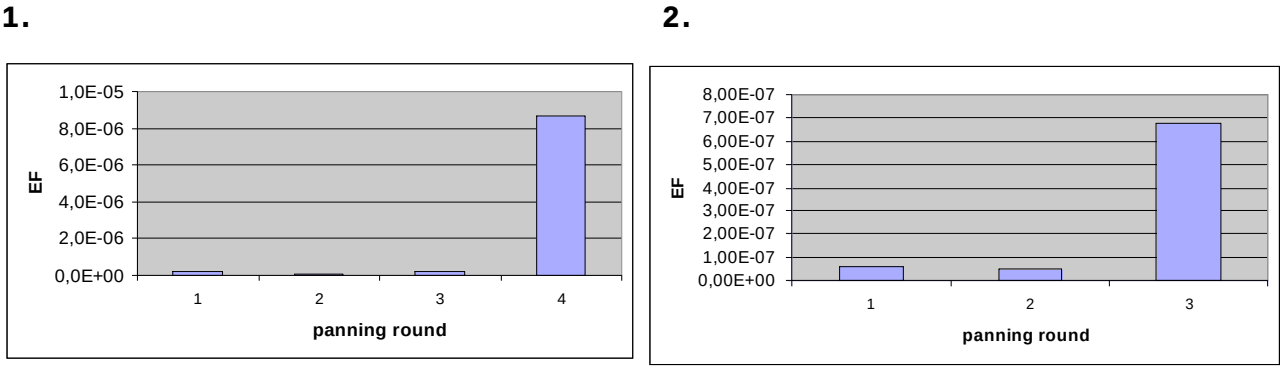


Figure 2.6 Enrichment of interacting abomasal phages during panning of the L4 infected and adult worm-infected abomasal cDNA libraries against respectively native L4 *Ostertagia* ES products (1) and adult *Ostertagia* ES products (2) (Enrichment factor (EF) = output phages/ input phages).

After the third selection round, 45 and 40 individual clones were respectively selected from the adult and L4 ESP screening. PCR analysis of these 85 clones indicated that 16 contained no insert. From the remaining phages, 48 were selected based on their insert size and submitted for sequencing. The Blast searches indicated that 34 clones contained a predicted bovine sequence, while 14 clones contained a predicted *O. ostertagi* sequence. The latter is likely to be a result of contaminating worm material in the abomasal tissue. From the 34 clones, only 5 contained a bovine sequence in the correct reading frame. The homologies are shown in table 2.2. They include a lysozyme, a beta-2-microglobulin, an adenylate cyclase stimulating G alpha protein (GNAS), a tubulin cofactor A and a cathepsin C. All the remaining clones contained a bovine sequence in the wrong reading frame, which would result in the expression of short random peptides with no specific homology. Furthermore, the specificity of the 5 remaining clones was evaluated on dot blot. They all reacted specifically with the spotted ES products but the positive spots have a different degree of intensity (Fig 2.7).

Table 2.2. List of abomasal peptides expressed by phages interacting with adult and L4 *Ostertagia* ES products.

Stage	Interacting clones	Genbank Accession number	Pept. length (AA)
Adult	*Bos taurus lysozyme C (precursor)*	**P04421**	156
Adult	*Bos taurus Beta-2-microglobulin (precursor) or lactollin*	**X69084**	127
Adult	*Bos taurus guanine nucleotide binding protein G(s), alpha subunit; Adenylate cyclase stimulating G alpha protein (GNAS)*	**X03404**	313
Adult	*Bos taurus tubulin cofactor A*	**X97224**	129
L4	*Bos taurus cathepsin C*	**BC102115**	39

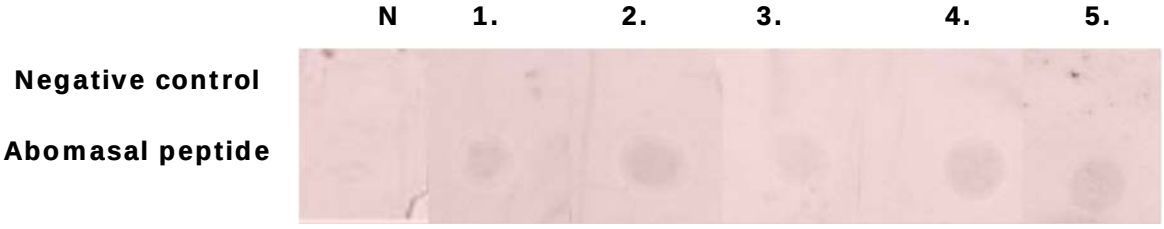


Figure 2.7 Dot Blot of the 5 remaining abomasal peptides with the negative control spot (Soybean trypsin inhibitor): (N) nonsense peptide (incorrect reading frame) (1) lysozyme, (2) beta-2-microglobulin, (3) GNAS, (4) a tubulin cofactor A and (5) cathepsin C.

The abomasal proteins, identified after panning against adult and L4 *O. ostertagi* ESPs, belong to a wide variety of cellular pathways. Lysozyme is a secreted enzyme with a bacteriolytic function (Irwin and Wilson, 1990). Beta-2-microglobulin is the beta-chain of MHC class I molecules where it has the role to stabilize the structure (Hoshi *et al*., 2000). The intracellular GNAS protein is the alpha subunit of G proteins that are essential for transducing stimulatory signals from surface receptors into intracellular responses (Nukada *et al*., 1986). Folding of microtubules, important cytoskeletal components, is coordinated by several chaperone proteins amongst which a tubulin cofactor A (Nolasco *et al*., 2005). Finally, cathepsin C is an intracellular lysosomal cysteine proteinase that plays an important role in protein degradation and the activation of proenzymes (Prin-Mathieu *et al*., 2002).

Our original objective was to identify the interacting molecules involved in the change of abomasal pH, immunomodulation, cell hyperplasia, etc. However, most of the proteins described above are not involved in such processes or are localized intracellularly. It is therefore unlikely that the interactions with *Ostertagia* ES material are relevant under *in vivo* conditions. The beta-2-microglobulin on the other hand is an extracellular protein and has a role in the host immune system. However, this protein is part of the MHC class I molecules which are important in the presentation of peptides derived from cytosolic pathogens. At the moment, there is no indication that *O. ostertagi* or any other gastrointestinal nematode interacts with this component of the immune system. For this reason the possible interaction between the beta-2-microglobulin and *Ostertagia* ES material was not further investigated.

Most of the protein receptors involved in the processes which *O. ostertagi* modulates during an infection are expected to be localized on the membrane surface of the cells (Klesius, 1993; Simpson, 2000). However, recent data indicated that the display on phages of transmembrane proteins such as receptor molecules, cell wall proteins or other structures embedded into membrane bilayers may be problematic (Rhyner et al., 2002, 2004; reviewed by Paschke, 2006) as is also reflected in our results. Successful display of foreign proteins requires expression of the fusion protein in *E. coli*, translocation to the periplasm and correct folding finalised by incorporation in the virions (Hufton *et al*, 1999). However, transmembrane proteins have a particular folding and a strong affinity for lipid bilayers. Therefore, the limited folding capacity of *E. coli* and the possibility that the phage coat protein fused to an insoluble transmembrane protein will not be translocated to the periplasm, could severely diminish or even stop the assembly of the phage. Such 'problem' phages would be quickly overgrown by other phages at each phage preparation step.

To circumvent this problem, a second experiment was set up, in which native membrane proteins of the abomasal epithelial cells were screened against an *O. ostertagi* phage display library. The adult *O. ostertagi* phage display library

was comprised of 1 x 10^6 clones in pG6A, pG6B and pG6C. Inserts ranging from 300 to 1700 bp were found in 80% of the clones by PCR screening with pG6 specific primers (Fig 2.8) (average insert size= 530 bp).

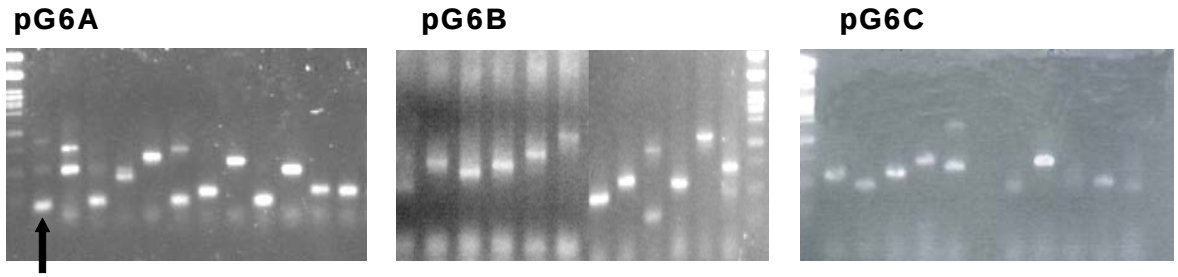


Figure 2.8 Adult *O. ostertagi* cDNA phage display libraries in pG6A, B and C phagemid vectors. Clones without a cDNA insert result in a PCR fragment of approximately 200 bp. One example is indicated with an arrow.

Approximately 4.074 x 10^6/ ml (total volume = 5 ml) abomasal epithelial cells were isolated with a dead/alive ratio of 40/60. 5 x 10^6 living cells were processed with the Cell Compartiment kit (Qiagen, Benelux BV.) and 216 µg membrane fraction was isolated (Fig 2.9).

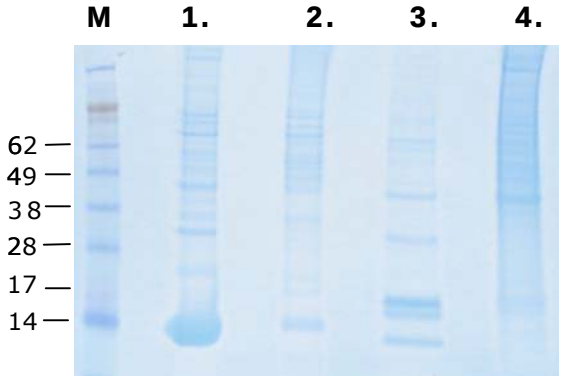


Figure 2.9 Subcellular fractionation of abomasal epithelial cells with respectively (1) cytosolic, (2) membrane, (3) nuclear and (4) cytoskeletal proteins

Subsequently, biopanning experiments were set-up to enrich potential *O. ostertagi* proteins with affinity for the abomasal cells or membrane fraction. Panning with the intact abomasal cells did not seem to enrich the *O. ostertagi* phage display library and hence the biopanning procedure was repeated with the membrane fraction of the abomasal epithelial cells. After 4 rounds of panning (Fig 2.10), 40 individual clones were selected at random and the insert size was determined by PCR-colony screening with pG6 specific primers. Eighteen clones did not contain an insert. Sequence analysis of the remaining clones and their predicted open reading frames revealed that none of the inserts was cloned in the correct reading frame. This resulted in the expression and display of very short peptides (maximum length of 23 amino acids) with no homology to any known nematode protein.

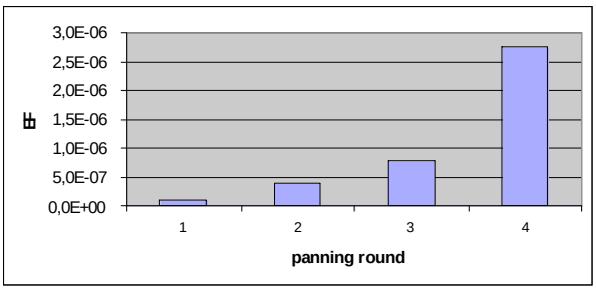


Figure 2.10 Enrichment of interacting *Ostertagia* phages during panning against native membrane proteins of the abomasal epithelial cells.

Additional tests with blotted membrane fraction probed with the three adult *O. ostertagi* phagemid libraries (data not shown) or native biotinylated ESPs (Fig 2.11) respectively, did not reveal any specific interactions. This could be attributed to the low concentrations of the interaction partners or to the low affinity of the studied ES-membrane protein interactions.

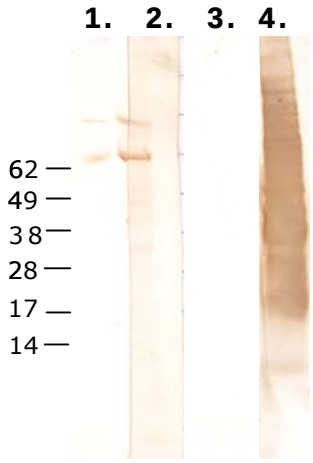


Figure 2.11 Western blot of membrane fraction of abomasal epithelial cells probed with biotinylated ESPs of adult *O. ostertagi* (2) with negative controls for endogenous biotines present in the membrane fraction(1) and adult ESPs (3). A positive controle (4) to confirm the biotinylation of the applied ES material was also included.

Although cDNA phage libraries were produced for both approaches, each panning round apparently has enriched for phages, which only displayed a very short random peptide. This seems to be an intrinsic problem with the phage display technology (Paschke, 2006). Due to the selection pressure, phage display vectors with short or defective inserts are more efficiently propagated than vectors containing longer open reading frames (Hust *et al*, 2006). This might be circumvented by the use of N-terminal phage display libraries, instead of the C-terminal library used in this study. An example of such a N-terminal system is the recently published Hyperphage, which uses a helper phage with a truncated *gIII* gene encoding the minor coat protein III (Hust *et al*., 2006). However, it is obvious that the full-length cDNAs fused at the N-terminal end cannot contain

stop codons. The cDNAs should therefore be produced with random oligos instead of a poly dT primer.

An additional problem encountered in our studies is the identification of irrelevant interactions, for example the interaction between ESPs and the bovine tubulin cofactor A. Although it is possible that the ESPs contain one or more intracellular components of the worm, which interact with the tubulin cofactor A, these molecules would never interact *in vivo*. Future selection experiments should therefore be made more specific and relevant. This could be done by reducing the number of possible interaction partners, for example a phage library solely containing secreted proteins of the parasite. A technique such as the signal sequence trap (SST) (Dalton *et al*., 2003) could be used to select and clone the cDNAs encoding secreted/membrane proteins.

In conclusion, although the phage display technology has shown great potential to study protein-protein interactions, it is now becoming clear that the methodology still has its limitations when used to perform large scale cDNA screens. However, in recent years many new phage display systems have been developed and many more will certainly emerge. It is therefore not unlikely that a system will be developed which would offer solutions to the problems we have encountered in this study.

4. References

Almeria, S., Canals,A., Zarlenga, D.S. and Gasbarre L.C. (1997). Isolation and phenotypic characterization of abomasal mucosal lymphocytes in the course of a primary *Ostertagia ostertagi* infection an calves. Vet Immunol Immunopath 57, 87-98.

Arap, M.A. (2005). Phage display technology: applications and innovations. Genet Mol Biol 28, 1-9.

Azzazy, H.M.E. and Highsmith, W.E. Jr. (2002). Phage display technology: clinical applications and recent innovations. Clin Biochem 35, 425-45.

Crameri, R. and Suter, M. (1993). Display of biologically active proteins on the surface of filamentous phages: a cDNA cloning system for selection of functional gene products linked to the genetic information responsible for their production. Gene 137, 69-75.

Dalton, J.P., Brindley, P.J., Knox, D.P., Brady, C.P., Hotez, P.J., Donnelly, S., O'Neill, S.M., Mulcahy, G. and Loukas, A. (2003). Helminth vaccines: from mining genomic information for vaccine targets to systems used for protein expression. Int J Parasitol 33, 621-40.

Eiler, H., Baber, W., Lyke, W.A. and Scholtens, R. (1981). Inhibition of gastric hydrochloric acid secretions in the rat given *Ostertagia ostertagi* (a gastric parasite of cattle) extract. Am J Vet Res 42, 498-502.

Fransen, M., Van Veldhoven, P.P.and Subramani, S. (1999). Identification of peroxisomal proteins by using M13 phage protein VI phage display: molecular evidence that mammalian peroxisomes contain a 2.4-dienoyl-CoA reductase. Biochem J 340, 561-568.

Fuh, G. and Sidhu, S.S. (2000). Efficient phage display of polypeptides fused to the carboxy-terminus of the M13 gene-3 minor coat protein. FEBS Letters 482, 231-234.

Fuh, G., Pisabarro, M.T., Li,Y., Quan, C., Lasky, L.A. and Sidhu, S.S. (2000). Analysis of PDZ domain-ligand interactions using carboxyl-terminal phage display. J Biol Chem 275, 21486-91.

Geldhof, P., Claerebout, E., Knox, D.P., Agneessens, J. and Vercruysse, J. (2000). Proteinases released in vitro by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. Parasitol 121, 639-647.

Hoshi, F., Nagai, D., Nakajima, Y., Higuchi, S. and Kawamura, S. (2000). Purification of bovine urinary β2-microglobulin and its biochemical characteristics. J Vet Med Sci 62, 867-74.

Hufton, S.E., Moerkerk, P.T., Meulemans, E.V., de Bruïne, A., Arends, J.W. and Hoogenboom, H.R. (1999). Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. J Immunol Methods 231, 39-51.

Hust, M., Meysing, M., Schirrmann, T., Selke, M., Meens, J., Gerlach, G.F. and Dübel S. (2006). Enrichment of open reading frames presented on bacteriophage M13 using hyperphage. Biotechniques 41, 335-42.

Irwin, D.M. and Wilson, A.C. (1990). Concerted evolution of ruminant stomach lysosomes. Characterisation of lysozyme cDNA clones from sheep and deer. J Biol Chem 265, 4944-52.

Jefferies, D. (1998). Selection of Novel Ligands from Phage Display Libraries: An Alternative Approach to Drug and Vaccine Discovery? Parasitol Today 14, 202-206.

Jespers, L.S., Messens, J.H., De Keyser, A., Eeckhout, D., Van Den Brande, I.,Gansemans, Y.G., Lauwereys, M.J., Vlasuk, G.P. and Stanssens, P.E. (1995). Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. Biotechn (N.Y.) 13, 378-382.

Klesius, P.H. (1993). Regulation of immunity to *Ostertagia ostertagi*. Vet Parasitol 46, 63-79.

McCafferty, J. and Johnson, K.S. (1996). Construction and screening of antibody display libraries. In: Kay, B.K., Winter, W. and McCafferty, J. (Eds.), Phage Display of Peptides and Proteins: a laboratory manual. Academic Press, Inc., 79-111.

McKellar, Q.A., Mostofa, M. and Eckersall, P.D. (1990). Stimulated pepsinogen secretion from dispersed abomasal glands exposed to *Ostertagia* species secretion. Res Vet Sci 48, 6-11.

Nolasco, S., Bellido, J., Gonçalves, J., Zabala, J.C. and Soares, H. (2005). Tubulin cofactor A gene silencing in mammalian cells induces changes in microtubule cytoskeleton, cell cycle arrest and cell death. FEBS Letters 579, 3515-3524.

Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Hirose, T, Inayama, S and Numa, S. (1986). Primary structure of the α-subunit of bovine adenylate cyclase-stimulating G-protein deduced from the cDNA sequence. FEBS Letters 195, 220-4.

Paschke, M. (2006). Phage display systems and their applications. Appl Microbiol Biotechnol 70, 2-11.

Prin-Mathieu, C., Le Roux, Y., Faure, G.C., Laurent, F., Béné, M.C. and Moussaoui, F. (2002). Enzymatic activities of bovine peripheral blood leukocytes and milk polymorphonuclear neutrophils during intramammary inflammation caused by lipopolysaccharide. Clin Diag Lab Imm 9, 812-7.

Rhyner, C., Kodzius, R. and Crameri., R. (2002). Direct selection of cDNAs from filamentous phage surface display libraries: potential and limitations. Curr Pharmacol Biotechnol 3, 13-21.

Rhyner, C.Weichel, M., Fluckiger, S., Hemmann, S. Kleber-Janke, T. and Crameri, R. (2004). Cloning allergens via phage display. Methods 32, 212-218.

Scott, I., Hodgkinson, S.M., Khalaf, S., Lawton, D.E.B., Collett, M.G., Reynolds, G.W., Pomroy, W.E. and Simpson, H.V. (1998). Infection of sheep with adult and larval *Ostertagia circumcincta*: abomasal morphology. Int J Parasitol 28, 1383-92.

Simpson, H.V., Simpson, B.H., Simcock, D.C., Reynolds, G.W. and Pomroy, W.E. (1999). Abomasal secretion in sheep receiving adult *Ostertagia circumcincta* that are prevented from contact with the mucosa. N Z Vet J 47, 20-4.

Simpson, H.V. (2000). Pathophysiology of abomasal parasitism: is the host or parasite responsible? Vet J 160, 177-91.

Smith, G.P. (1985). Filamentous fusion phage: novel expression vectors that display cloned antigens on the virions surface. Sci 228, 1315-1317.

Smith, G.P. and Scott, J.K. (1993). Libraries of peptides and proteins displayed on filamentous phage. Methods Enzymol 217, 228-57.

Viaene, A., Crab, A., Meiring, M., Pritchard, D. and Deckmyn, H. (2001). Identification of a collagen-binding protein from *Necator americanus* by using a cDNA-expression phage display library. J Parasitol 87, 619-625.

Wildblood, L.A., Kerr, K., Clark, D.A.S., Cameron, A., Turner, D.G. and Jones, D.G. (2005). Production of eosinophil chemoattractant activity by ovine gastrointestinal nematodes. Vet Imm Immunopath 107, 57-65.

Chapter 3

Identification and characterization of a novel specific secreted protein family for selected members of the subfamily Ostertagiinae (Nematoda)

* Based on the manuscript: Saverwyns, H., Visser, A., Nisbet, A.J., Peelaers, I., Gevaert, K., Vercruysse, J., Claerebout, E. and Geldhof, P. (2008). Identification and characterization of a novel specific secreted protein family for selected members of the subfamily Ostertagiinae (Nematoda). Parasitology 135, 63-70.

1. Introduction

Ostertagia ostertagi is a highly pathogenic gastrointestinal nematode, which infects cattle in temperate climate regions. During its development, the parasite induces several morphological, physiological and biochemical changes in the abomasum of the host. These changes result in tissue damage, a substantial increase in the pH of the abomasal contents, loss of functional activity of parietal cells, hypoalbuminaemia and hypergastrinaemia (McKellar, 1993). Also, *O. ostertagi* induces a strong Th2 type immune response, accompanied by accumulation of local antibodies, mast cells and eosinophils (Claerebout and Vercruysse, 2000). Although the mechanisms or processes used by the parasite to change the abomasal environment are largely unknown, parasite excretory-secretory products (ESPs) are considered to play an important role (Klesius, 1993; Simpson *et al.,* 1999; Simpson, 2000). ESPs comprise all of the material released by the parasites *in vitro* and presumably also *in vivo*. ESPs are often stage-specific and may be derived from the parasite surface, from specialized secretory glands or as by-products of parasite digestion (Knox, 2000). In an attempt to gain insight in the composition of *O. ostertagi* ESPs, Vercauteren *et al.* (2003) immuno-screened cDNA libraries of different life cycle stages of *O. ostertagi* with polyclonal rabbit serum raised against ESPs. This approach led to the identification of 15 secreted proteins, of which many were nematode specific but with an unknown function. The possible role of ES antigens as vaccine candidates also makes them particularly interesting. An ES antigen fraction purified from adult *O. ostertagi* ES material by thiol sepharose chromatography has previously been shown to partially protect cattle against challenge infection in two independent vaccination experiments (Geldhof *et al*., 2002; 2004). Mass spectrometric (MS) analysis indicated that the most abundant antigens present in this fraction were two activation-associated secreted proteins, termed Oo-ASP1 and Oo-ASP2 (Geldhof *et al*., 2003). A recent reanalysis of these MS data by comparison against the currently available *O. ostertagi* expressed sequence tag (EST) dataset identified a previously undescribed nematode-specific protein family. In the present study, we report the further characterization of this novel nematode specific secreted protein family present in selected members of the subfamily Ostertagiinae, in specific *O. ostertagi* and *Teladorsagia circumcincta*. The transcription and expression pattern of its members and the phylogenetic relationship with the SCP/Tpx-1/Ag5/PR-1/Sc7 protein family were analysed.

2. Material and methods

2.1. Bioinformatics

The full-length sequence of *Oo-al1* (*O. ostertagi* ASP-like protein 1) was obtained by means of the BD Marathon® cDNA amplification kit (BD Clontech).

The 5'-rapid amplification of cDNA ends (RACE) PCR was performed on an aliquot from an adult *O. ostertagi* cDNA library using the AP1 primer and a gene specific primer OoAL1RTrev (5'-KCTTGTTATTRTGAATCCGTCA-3') or OoAL2RTrev (5'-GTCGTGAATGGGCTTCTGTT-3'), as recommended by the manufacturer. Sequencing was carried out at the Center for Medical Genetics (University Hospital Ghent) by use of the dideoxy chain terminator method (Big Dye™ terminator v3.1, Applied Biosystems) and the ABI3730xl genetic analyzer (Applied Biosystems). Database searches for homologues in other nematode species were carried out *via* NEMBASE (http://zeldia.cap.ed.ac.uk/ncbi_blast.html) and NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment and analyses of these nucleotide and deduced amino acid sequences were conducted using the program DNASTAR (DNAstar Inc). N-glycosylation and phosphorylation sites were predicted with PROSITE (www.expasy.ch/tools/scanprosite), while signal peptides were detected with SignalPV1.1 (www.cbs.dtu.dk/services/SignalP). Protein domain searches were also performed using the NEMBASE2 database (http://zeldia.cap.ed.ac.uk/nematodeESTs/search_domains.php). Multiple alignments were made using CLUSTALW (http://www.ebi.ac.uk/clustalw), and phylogenetic analysis was carried out using the program Mega v.3.1 (Kumar *et al.,* 2004). The neighbor-joining method was employed and the consensus tree (unrooted) selected from the analysis.

2.2. Genomic DNA organisation

Genomic DNA was extracted from pooled *O. ostertagi* using the NucleoSpin® tissue kit (Macherey-Nagel). The PCR mixture (25 µl) consisted of 2 µl template genomic DNA (1/10), 0.8 µM of forward primer, 0.8 µM of reverse primer, 1 U of Platinum® *Taq* DNA polymerase High Fidelity, 0.4 mM dNTP mixture, 2 mM $MgSO_4$ and 2.5 µl 10 x High Fidelity PCR Buffer (Invitrogen). The various primer pairs used in this protocol can be found in table 3.1. The PCR was performed using the following conditions: 4 min at 94°C, followed by 35 cycles of 60 s at 94°C, 60 s at 60°C, 2 min 30 s at 68°C, and a final extension of 10 min at 68°C. The amplicons were subjected to electrophoresis in 1.5 % agarose gels, subsequently excised and purified using NucleoSpin® Extract II kit (Macherey-Nagel). The purified amplicons were cloned into the pGEM®-T Easy vector and *Escherichia coli* (strain DH5α; Invitrogen) was transformed. Sequencing was performed as described in the previous section.

Table 3.1 The various primers used for obtaining the genomic DNA organisation of the *Oo-al* genes (R = A,G and M = A, C).

name	DNA sequence (5' to 3')
OstcloneForw	RCGCGAACCCATTCCMTA
RT rev	ACGGCAGTCTTCAACGTTTC
OoAL1rev	AACAAGCGTTCTGCGCTTT
HdiStartF	ATGAATCTGCACACCCCGTC
Hdi1700F	AAATATGCGGGCGGATAAG
Hdi2000F	AATATGCGGGCGGATAAGTT
OstgDNAForw	ATAGTTGTGCGCTTGCAACA
OstgDNArev	AGCATGAAGTGTTTCACCATGT
OstcloneSalIpET	GTCGACCATGTATTGGTAATT
OoPR1(2000)F	GCGCTGAAAAGCTCTCCTTA
OoPR1F	AACATGGTGAAACACTTCATGC
OoAL1 2500Rev	GCCATTCAAAAGGTGGAAAA
OoPRstartRev	TCATACTGGGATGAGAGCTTG

2.3. Stage specific transcription

A PCR approach was used to investigate the transcription of *Oo-al* and *Tc-al* (*T. circumcincta* ASP-like protein). For *O. ostertagi*, RNA extraction and cDNA preparation were performed as described previously (Van Zeveren *et al*., 2007). The reaction mixture (25 µl) consisted of a master mix with *Taq* DNA polymerase (Invitrogen), dNTP, 1.5 mM $MgCl_2$, 0.4 µM of both the forward (RTforw: 5'-CCCTAAAGAGTCGCCAACAAG-3') and reverse gene-specific primer (OoAL1RTrev, OoAL2RTrev) and 1 µl of the cDNA (1/20) of the different developmental stages of *O. ostertagi*. The PCR started with a denaturation step at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C to 68°C (depending on the primer combination) for 30 s, 72°C for 30 s, and a final extension of 10 min at 72°C. To correct for sample-to-sample variation during RNA isolation and reverse transcription step, the transcription profile of the reference gene (*rpl13*) was also used for comparison. The amplicons were electrophoresed in 2 % agarose. Bands were photographed using the Quantity One 4.5.1 Chemidoc EQ™ Software System (Bio-Rad, CA, USA).

For *T. circumcincta*, single-stranded cDNA (ss-cDNA) was synthesized from mRNA isolated from *T. circumcincta* eggs, infective L3, L4 (harvested 7 days *post infection*) and adults (harvested at 28 days) as described previously (Redmond *et al.*, 2006). Approximately 5 ng of ss-cDNA were used as template in the semi-quantitative PCR employing specific, internal primers (Tc_Al_For: 5'-CTACGATATGCTGCTTCGTCTT-3'; Tc_Al_1_Rev: 5'-GCACCCTTCAAGGTTTCATCC-3' and Tc_Al2b_Rev: 5'-ACATTCATCGACCTGGTTCC-3') for each of Tc-al1 and Tc-al2. The following cycling conditions were used: 94 °C for 5 min, followed by 32 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 1 min, with a final extension at

72 °C for 7 min. Equal loading and integrity of each of the ss-cDNA preparations was verified by PCR using primers designed to amplify a 320 bp fragment of the *T. circumcincta* beta-tubulin gene. Amplicons were separated in a 1.5 % (w/v) agarose gel and detected by staining with GelRed™ nucleic acid gel stain (Biotium, Hayward, USA).

2.4. Recombinant expression of Oo-AL1

The *Oo-al1* cDNA (381 bp) was first cloned into the pGEM®-T-Easy vector (Promega Corporation), following PCR using primers containing the restriction sites BamHI and SalI (OoALBamHI: 5'-GGATCCGACTGCAACGAGAACAT-3', OoALSalI: 5'-GTCGACCTACATGTATTGGTAATT-3'). The OoAL- pGEM®-T-Easy plasmid DNA was purified with Qiagen plasmid Midi kit (Qiagen) and digested with both the restriction enzymes (Invitrogen). The digested *Oo-al1* fragment was gel-purified and unidirectionally ligated in the expression vector pGEX-6P-1. Competent cells of *E. coli* strain BL21 (DE3; codon plus) were transformed and transformants selected. The DNA sequence and correct reading frame were verified. Clones containing the *Oo-al1*-pGEX-6P-1 construct were grown in 2 x YT broth at 37°C to an optical density (600 nm) of 0.6, after which recombinant Oo-AL1 protein (rOo-AL1) synthesis was induced by the addition of 0.1 mM isopropyl-beta-D-thiogalactopyranoside (2 h at 37°C). Cell lysis and purification of the protein on glutathione agarose beads were performed according to an established protocol (Frangioni and Neel, 1993). The glutathione S-transferase affinity tag was removed by PreScission protease cleavage (GE Healthcare). The protein concentration and purity were determined by SDS-PAGE and subsequent Coomassie blue staining, by comparison with a known bovine serum albumin standard (Pierce).

2.5. Immunization of rabbit

A New Zealand white laboratory rabbit (4 months) was immunised subcutaneously with ~ 30 µg of purified recombinant Oo-AL1, three times with a two week interval. Each dose was supplemented with 5 µg Quil A (Superfos). Two weeks after the final immunisation, the serum was collected.

2.6. Affinity purification of antibodies from rabbit serum

To diminish cross-reactivity, serum antibodies were affinity purified to rOo-AL1. Approximately 400 µg of rOo-AL1 was separated on SDS-PAGE and transferred to a polyvinyldine difluoride (PVDF) membrane (Immobilon®, Milipore). The Oo-AL1 region was excised and the PVDF strip was blocked with 10 % horse serum (HS) in PBS containing 0.05 % Tween 20 (Sigma) (PBST). The

blot was incubated overnight at 4°C with the serum (diluted 1:40 in PBST+2 % HS) and then washed extensively with PBST (3 x 5 min at room temperature). Bound antibodies were eluted by incubating the blot with 5 ml of 5 mM glycine, 0.5 M NaCl, pH 2.5 for 5 min. The eluted antibodies were neutralised immediately with 100 µl 1M Tris pH 8.5. Finally HS (final concentration of 5 %) was added to stabilize the purified antibodies (henceforth referred to as α-Oo-AL1 antibody).

2.7. SDS-PAGE and Western blot

Somatic extracts (EX) from the L4 and adult life stage were prepared as described by De Maere *et al*. (2002). ESPs from L4s or adults were collected as described by Geldhof *et al*. (2000). Ten micrograms of each EX and ESPs and 5 µl of the positive control (rOo-AL1) were separated on NuPAGE® 10 % Bis-Tris Gels (Invitrogen) under reducing and non-reducing conditions and subsequently blotted on to a PVDF membrane. Non-specific binding was blocked with 10 % HS (in PBST). The blots were incubated with the α-Oo-AL1 antibody at 4°C overnight. Subsequently, the blots were washed extensively with PBST (3 x 5 min at room temperature) and incubated with affinity purified goat anti-rabbit IgG (Sigma, 1:5000 in PBST with 2 % HS) conjugated with horse-radish peroxidase and washed. The substrate 3.3 diaminobenzidine tetrachloride in PBS and 0.02 % H_2O_2 was used to detect bands.

3. Results

Reanalyses of the previously obtained MS data for the ES-thiol fraction (Geldhof *et al*., 2003) against the *O. ostertagi* EST dataset resulted in a significant hit with EST contig number OOC00098. This contig represents 2 EST sequences (BG733966, BG733967), each encoding a predicted protein of 149 amino acids (aa). Protein domain searches with this sequence revealed a low but significant homology (E-value = 0.65 with a significant E-value cut-off level ≤ 1) with the SCP/Tpx-1/Ag5/PR-1/Sc7 protein motif. Although this motif is also present in the activation associated secreted protein (ASP) family of nematodes, sequence analysis indicated that this *O. ostertagi* ESP was distinct from all of the previously identified/characterized ASPs. Therefore, the protein was named the *O. ostertagi* ASP-like protein Oo-AL1, which has an estimated molecular weight of 16.6 kDa and a predicted pI of 6.32.

PCR amplification from the *Oo-al1* cDNA from different parasite life stages revealed the presence of an additional *Oo-al* transcript in *O. ostertagi*, termed *Oo-al2*. The protein encoded by this transcript had a 81.6 % sequence identity to protein Oo-AL1, has an estimated molecular weight of 8.5 kDa and a predicted pI of 8.33. Using the *O. ostertagi Oo-al* sequences to query all nematode genome and EST databases available, six similar EST sequences were identified in *T.*

circumcincta. The AL proteins of *T. circumcincta* are encoded by 2 EST clusters. The first cluster TDC00472 consisted of 5 ESTs (CB037847, CB037904, CB038060, CB038565, CB039149) which code for a long variant of Tc-AL1 (163 aa) of ~ 17.5 kDa. The other EST cluster TDC00684 contained one EST (CB038818) which was 498 nucleotides (nt), coding for a shorter protein Tc-AL2 (114 AA) of 13 kDa. An alignment of the AL sequences for *O. ostertagi* and *T. circumcincta* is shown in Fig. 3.1. The proteins inferred from these *Teladorsagia* ESTs exhibit 48.7-58.7 % sequence identity with the Oo-AL proteins. One putative N-linked glycosylation site (Asn-Leu-Thr-Tyr) was conserved in these 4 AL proteins from *Ostertagia* and *Teladorsagia* and is located between amino acids 55 and 57 (Fig. 3.1, marked with a star). Each of these proteins contained a signal peptide motif (Fig. 3.1, marked with an arrow), suggesting that these proteins are genuinely secreted.

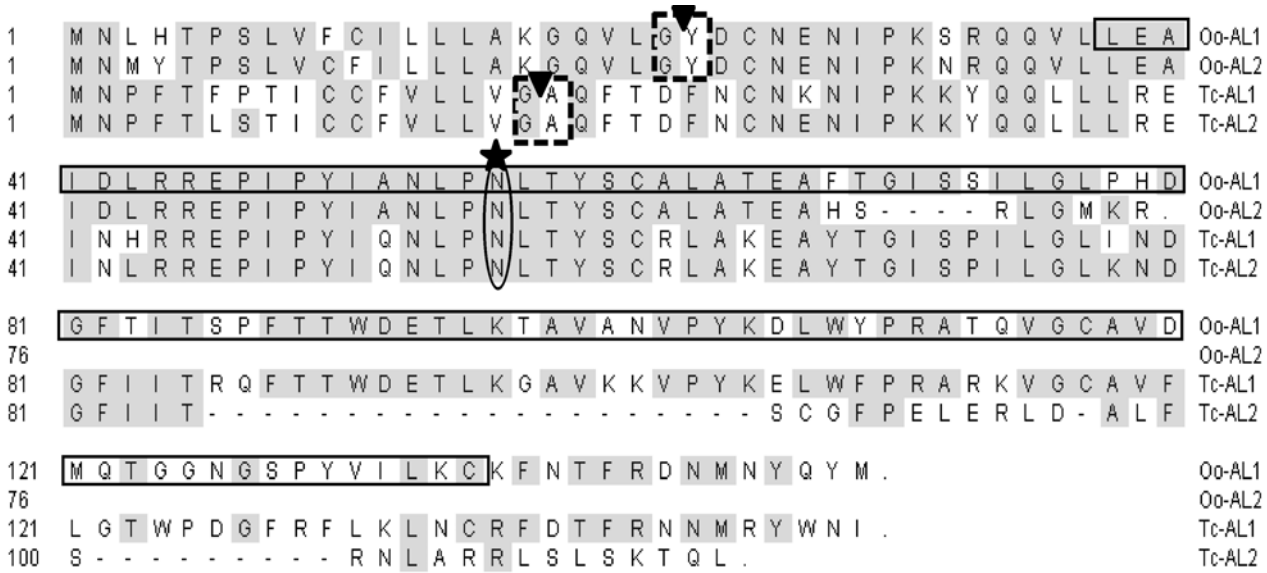


Figure 3.1 Alignment of the predicted amino acid sequences of the AL proteins (Oo-AL1, Oo-AL2, Tc-AL1, Tc-AL2). The cleavage site of the N-terminal signal peptide is indicated by an arrow head (▼). The putative N-linked glycosylation site is marked with a star (★). The SCP-like extracellular protein domain in Oo-AL1 is marked with a box.

The *Oo-al2* cDNA sequence is 98.5 % identical to *Oo-al1*, except for 6 nucleotide polymorphisms and an indel of 58 nucleotides at position 199. To investigate whether the transcripts were encoded by two different genes or were the result of alternative splicing, PCR was performed on genomic DNA from pooled and individual *O. ostertagi* adults to isolate Oo-AL encoding genes. Three predicted *Oo-al* genes (AM712631, AM712632 and AM712633) of ~ 2360, 3000 and 3500 bp, respectively, were isolated. Each gene had a similar structure and contained 7 exons and 6 introns (Fig. 3.2). The differences between the three genes related mainly to intron 3. The exon sequences of all the genes were almost identical to each other, and were presented to encode isoforms of the Oo-AL1 protein. None of these three genes encoded the Oo-AL2 protein. Based on these results, it is unlikely that Oo-AL2 is due to alternative splicing. The cDNA sequence of *Oo-al2* differed from that of *Oo-al1* in the lack of the last 58 nucleotides of exon 4. However, no different donor/acceptor splice sites could be identified in exon 4, which could explain this alternative truncation.

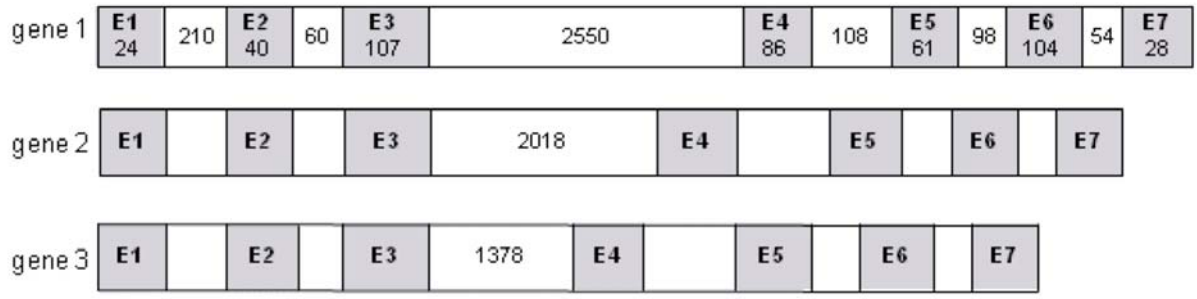


Figure 3.2 Genomic organisation of Oo-al genes. Length (bp) of introns and exons is indicated. Only the length of intron 3 differs among the 3 genes.

The analysis of the *O. ostertagi* genes suggests that the two AL proteins identified in the *T. circumcincta* EST dataset are encoded by one or more genes with a similar structure to those of *O. ostertagi*. The part of the protein missing in the short Tc-AL variant matches the sequence encoded by exon 5, indicating (in contrast to the situation in *O. ostertagi*) that the short Tc-AL2 protein might indeed be a splice variant of the gene encoding the full Tc-AL1 protein. The alternative splicing of exon 5 is inferred to result in a frame shift and an earlier termination of the translation.

As an outgroup could not be accurately defined, an unrooted phylogenetic tree was constructed to display visually the relationship of the AL proteins with other SCP-like extracellular proteins (Pfam PF00188) from various organisms. This protein family is characterised by a high diversity in protein sequence and function among its members (reviewed by Van Loon and Van Strien, 1999). In some parasites, such as hookworms, *Haemonchus contortus* and *O. ostertagi*, members of this protein family have proven their potential as vaccine antigens (Schallig *et al*., 1997; Geldhof, *et al.* 2002, 2003, 2004; Bethony *et al*., 2005).

The phylogenetic analysis revealed a close relationship of the AL proteins representing *Ostertagia* and *Teladorsagia*, which clustered with (previously characterised) ASPs but represented a distinct group (Fig. 3.3).

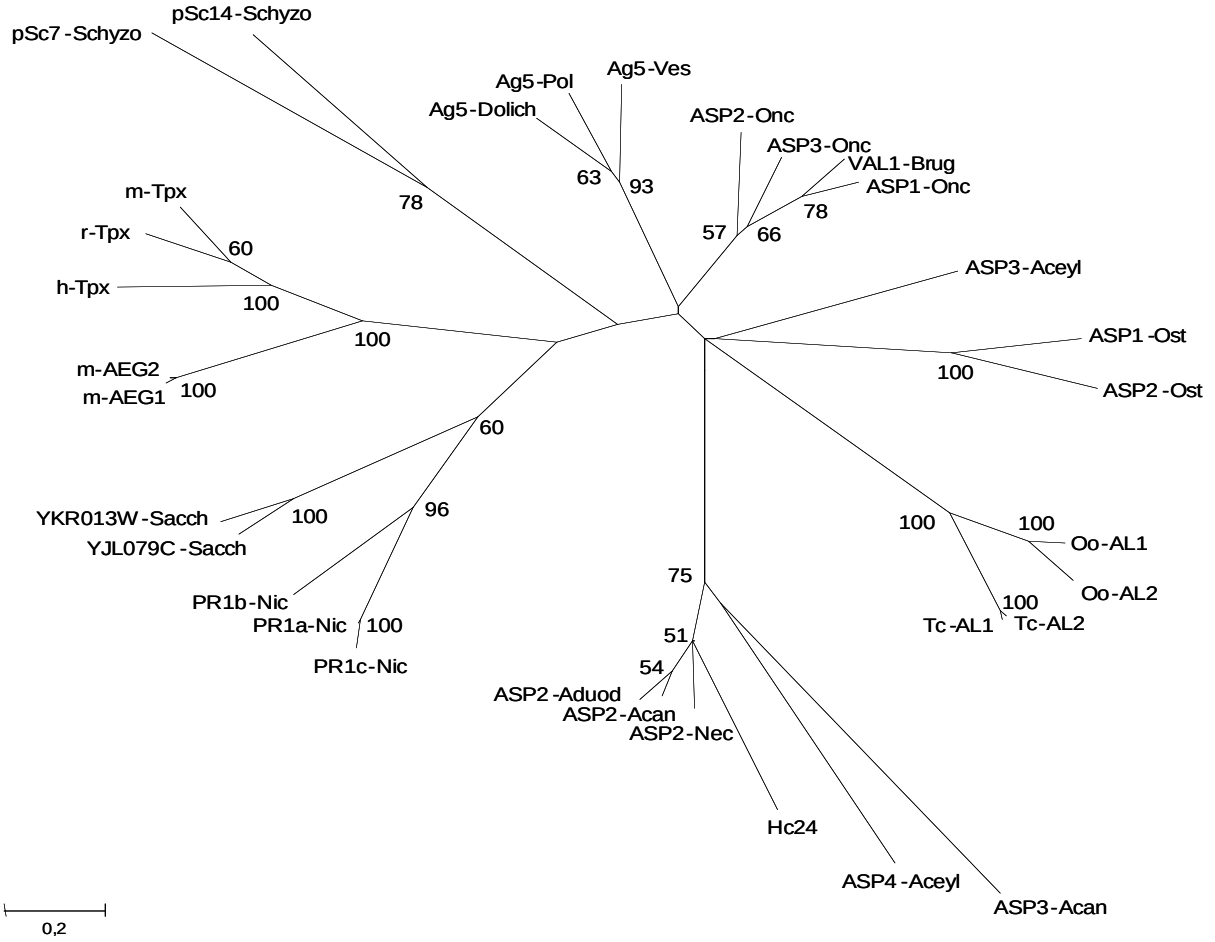


Figure 3.3 Unrooted phylogenetic tree showing the relationship of Oo-AL1, Oo-AL2 and other SCP-like proteins from different species such as testis specific proteins from humans (h-Tpx = X95237), mouse (m-Tpx = NP033446), rat (r-Tpx = AAD48090); mouse acidic epididymal glycoprotein-like molecules (m-AEG1 = AAA37185, m-AEG2 = AAA37186); *Schizophyllum commune* (pSc14-Schyzo = M81723, pSc7-Schyzo = M81722); *Saccharomyces cerevisiae (*YKR013W-Sacch = Z28238; YJL079C-Sacch = AY558306); *Nicotiana tabacum* pathogen related proteins (PR1a-Nic = X06361, PR1b-Nic = X17680, PR1c-Nic = X17681); vespid venom allergen 5 from *Vespula vulgaris* (Ag5-*Ves* = AAA30333), *Dolichovespula arenaria* (Ag5-*Dolich* = AAA28303), *Polistes annularis* (Ag5-*Pol* = AAA29793); *Brugia malayi* venom allergen antigen-like protein 1 (VAL1-Brug = AAB97283); ASPs from *Onchocerca volvulus* (ASP1-Onc = AAB69625, ASP2-Onc = AAP06732, ASP3-Onc = AAB97282), *O. ostertagi* (ASP1-Ost = CAD23183, ASP2-Ost = CAD56659), *Haemonchus contortus* (*Hc*24 = AAC47714), *Ancylostoma ceylaticum* (ASP3-Acetyl = AAR03712, ASP4-Acetyl = AAR03713), *A. caninum* (ASP2-Acan = AAC35986, ASP3-Acan = AAO63575), *A. duodenale* (ASP2-Aduod = AAP41951), *Necator americanus* (ASP2-Nec = AAP41952) and ASP-like proteins from *O. ostertagi* (Oo-AL1 = AM712629, Oo-AL2 = AM712630) and *T. circumcincta* (Tc-AL1 = TDC00472, Tc-AL2 = TDC00684). Bootstrap values of >50 are indicated.

The transcription of the AL protein was investigated in both *O. ostertagi* and *T. circumcincta* by PCR. Specific primers for each gene were used on cDNA from the different life stages. The results are shown in Fig. 3.4. The *Oo-al1* transcript was detected in the L4 stage and adult females and males of *O. ostertagi*. In *T. circumcincta*, the *Tc-al1* transcript was present from the exsheathed L3 stage onwards, with the highest level of transcription in the L4. The PCRs for the shorter AL transcript forms (*Oo-al2* and *Tc-al2*) revealed very faint bands, suggesting a low level of transcription for both of these forms (results not shown).

Figure. 3.4 (A) Stage specific transcription pattern of *Oo-al1* and the reference gene *rpl13* in the L3 stage with (L3+) and without sheath (L3-), the L4 stage, the adult females (F) and males (M) (BL=blank). (B) Stage specific transcription of Tc-al1 and the reference gene *β*-tubulin in eggs, exsheated L3s (L3-), L4 and adult stages.

The expression pattern for the AL proteins in *O. ostertagi* was also analysed on Western blots of somatic EX and ESPs from L4 and adult *O. ostertagi* using the α-Oo-AL1 antibody probe. The blots inferred the presence of an Oo-AL protein in the ESPs of adult worms. A band of ~ 14 kDa was detected under reducing conditions (Fig. 3.5, panel A). The Western blot of the non-reduced adult ESPs revealed a higher band, suggesting dimerisation of the OoAL proteins under native conditions (Fig. 3.5, panel B). The Oo-AL protein could not be detected on the Western Blots of the somatic extracts of the L4 and adult life stages (data not shown). The specific anti-OoAL-1 antibody probe was also used in an attempt to detect the corresponding native protein on sections made from the adult life stages of *O. ostertagi* (*cf*. Geldhof *et al*., 2005), but immunofluorescent labelling of Oo-AL1 was not detected (results not shown). This result may be attributable to insufficient amounts of Oo-AL1 to be detectable on the cross sections and corresponded to the Western blot analysis of somatic EX.

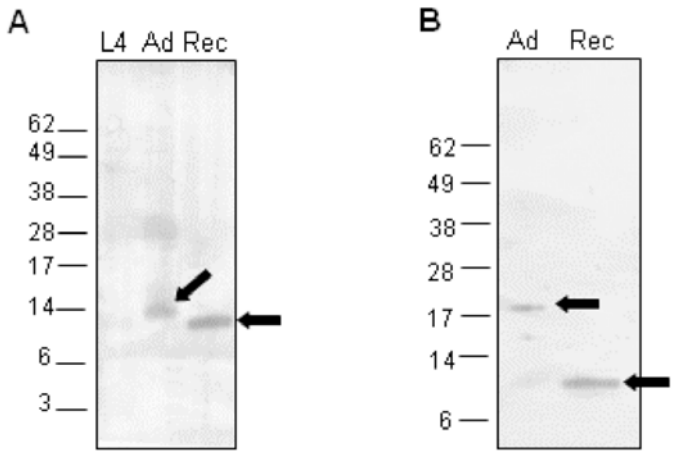


Figure 3.5 (A) Western blot (reducing conditions) of L4 ESPs, adult ESPs and rOo-AL1 probed with α-Oo-AL1 antibody. (B) Western blot (non-reducing conditions) of adult ES and rOo-AL1 probed with the same antibody.

4. Discussion

The present study provides a first insight into a novel nematode secreted protein family. We have named them the ASP-like (AL) protein family, because they contain the SCP/TPX-1/Ag5/PR-1/Sc7 protein domain (Pfam PF00188, InterPro domain IPR001283) which typifies the large nematode activation-associated secreted protein family (ASP). In *O. ostertagi*, the *Oo-al1* transcript was detectable from the L4 stage onwards, whereas in *T. circumcincta* the *Tc-al1* transcript was present from the exsheathed L3 stage onwards and was highest in the L4 larval stage.

There are at least three different genes encoding AL proteins in *O. ostertagi*. Surprisingly, all of these genes seem to code for the same protein, Oo-AL1. A gene encoding the shorter Oo-AL2 version could not be identified. The reason for this is unclear. Either Oo-AL2 is the product of a rare allele present in the population or Oo-AL2 is an isoform of Oo-AL1. The latter may be the case for the two AL variants in *T. circumcincta*, where Tc-AL2 lacks the amino acids encoded by exon 5. Alternative splicing, where an entire exon is deleted, is widely used by organisms to increase their proteome diversity (see Nagasaki *et al*., 2005). However, the situation for Oo-AL2 is different. The protein seems to be truncated within exon 4. This type of splicing with intron/exon isoforms has been reported previously in *Caenorhabditis elegans* and *C. briggsae* (see Kent and Zahler, 2000; Nagasaki *et al*., 2005), but requires alternative donor and acceptor splice sites. In this case, it would require a new 5' splice site in exon 4. Although the nucleotide frequencies adjacent to the 5' and 3' splice sites in *O. ostertagi* have not been studied in detail, they are likely to be conserved in relation to other nematodes such as *C. elegans*. Multiple studies have shown that the canonical 5' GT splice site in *C. elegans* is highly conserved (Kent and Zahler, 2000; Sheth *et*

al., 2006). This site is also present at the 5' end of each intron in the genes encoding ALs in *O. ostertagi*. However, no such alternative 5' GT splice site is present in exon 4, such that it is still unclear from which gene Oo-AL2 is derived.

The function of the AL proteins is presently unknown. The SCP/TPX-1/Ag5/PR-1/Sc7 protein domain is found in a broad range of proteins, such as the mammalian sperm-coating glycoproteins, cysteine rich secretory proteins, testis-specific proteins, venom antigens, plant pathogen related proteins (PR-1), lizard helothermine and some yeast proteins (reviewed by Van Loon and Van Strien, 1999). The suggested functions of these proteins are equally diverse. The mouse TPX-1 (also called CRISP-2) is thought to be involved in sperm maturation and possesses lytic enzymatic activities for the degradation of egg structures during fertilization (Kasahara *et al*., 1989). The PR-1 family is induced under stress conditions, like plant responses to pathogens and tumor growth (Dixon *et al*., 1991; Durrant and Dong, 2004; Basse, 2005). Helothermine is a toxin that blocks ryanodine receptors, whereas venom allergen 5 is a potent allergen that can induce allergic reactions in humans (Lu *et al*., 1993). The function of the nematode ASPs and ASP-like proteins is still unknown. It has been postulated that ASPs take part in the infection process and the transition to parasitism (Hawdon *et al*., 1996, 1999; Murray *et al*., 2001; Moser *et al*., 2005).

The database searches conducted herein indicated that this novel protein family is specific to the subfamily Ostertagiinae (order Strongylida). No homologous AL-like proteins were identified in any other nematode species, both parasitic and free-living. This information suggests a key functional role for the AL proteins in the life cycle of these abomasal parasites. The uniqueness of this group of proteins is also consistent with the outcome of a large scale transcriptomic analysis of the phylum Nematoda, showing that many genes containing the SCP/TPX-1/Ag5/PR-1/Sc7 domain have undergone "lineage-specific amplification and divergence" within the superfamily Strongyloidea (Parkinson *et al*., 2004). Furthermore, the AL sequences from *O. ostertagi* and *T. circumcincta* show notable sequence diversity between the species and a slightly different transcription pattern, which could relate to an adaptation to cattle and small ruminants, respectively.

5. References

Basse, C. W. (2005). Dissecting defense-related and developmental transcriptional responses of maize during *Ustilago maydis* infection and subsequent tumor formation. Plant Physiol 138, 1774-1784.

Bethony, J., Loukas, A., Smout, M., Brooker, S., Mendez, S., Plieskatt, J., Goud, G., Bottazzi, M. E., Zhan, B., Wang, Y., Williamson, A., Lustigman, S., Correa-Oliveira, R., Xiao, S. and Hotez, P. J. (2005). Antibodies against a secreted protein from hookworm larvae reduce the intensity of hookworm infection in humans and vaccinated laboratory animals. FASEB J 12, 1743-1745.

Claerebout, E. and Vercruysse, J. (2000). The immune response and the evolution of acquired immunity against gastrointestinal nematodes in cattle. Parasitol 120, S25-S40.

De Maere, V., Vercauteren, I., Saverwyns, H., Claerebout, E., Berx, G. and Vercruysse, J. (2002). Identification of potential protective antigens of *Ostertagia ostertagi* with local antibody probes. Parasitol 125, 383-391.

Dixon, D. C., Cutt, J. R. and Klessig, D. F. (1991). Differential targeting of the tobacco PR-1 pathogenesis-related proteins to the extracellular space and vacuoles of crystal idioblasts. The EMBO J 10, 1317-24.

Durrant, W. E. and Dong, X. (2004). Systemic acquired resistance. Ann Rev phytopathol 42, 185-209.

Frangioni, J. V. and Neel, B. G. (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. Analyt Biochem 210, 179-187.

Geldhof, P., Claerebout E., Knox, D. P., Agneessens J. and Vercruysse, J. (2000). Proteinases released *in vitro* by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. Parasitol 121, 639-647.

Geldhof, P., Claerebout, E., Knox, D., Vercauteren, I., Looszova, A. and Vercruysse, J. (2002). Vaccination of calves against *Ostertagia ostertagi* with cysteine proteinase enriched protein fractions. Parasite Immunol 24, 263-270. Geldhof, P., Vercauteren, I., Gevaert, K., Staes, A., Knox, D. P., Vandekerckhove, J., Vercruysse, J. and Claerebout, E. (2003). Activation-associated secreted proteins are the most abundant antigens in a host protective fraction from *Ostertagia ostertagi.* Mol Biochem Parasitol 128, 111-114.

Geldhof, P., Vercauteren, I., Vercruysse, J., Knox, D. P., van den Broeck, W. and Claerebout, E. (2004). Validation of the protective *Ostertagia ostertagi* ES-thiol antigens with different adjuvantia. Parasite Immunol 26, 37-43.

Geldhof, P., Whitton, C., Gregory, W. F., Blaxter, M. and Knox, D. P. (2005). Characterisation of the two most abundant genes in the *Haemonchus contortus* expressed sequence tag dataset. Int J Parasitol 35, 513-522.

Hawdon, J. M., Jonest, B. F., Hoffman, D. R. and Hotez, P. J. (1996). Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. J Biol Chem 271, 6672-6678.

Hawdon, J. M., Narasimhan, S. and Hotez, P. J. (1999). Ancylostoma secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. Mol Biochem Parasitol 30, 149-165.

Kasahara, M., Gutknecht, J., Brew, K., Spurr, N. and Goodfellow, P. N. (1989). Cloning and mapping of a testis-specific gene with sequence similarity to a sperm-coating glycoprotein gene. Genomics 5, 527-534.

Kent, W. J. and Zahler, A.M. (2000). Conservation, regulation, synteny, and introns in a large-scale *C. briggsae-C. elegans* genomic alignment. Genome Res 10, 1115-1125.

Klesius, P. H. (1993). Regulation of immunity to *Ostertagia ostertagi*. Vet Parasitol 46, 63-79.

Knox D. P. (2000). Development of vaccines against gastrointestinal nematodes. Parasitol 20, S43-S61.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Brief Bioinform 5, 150-163.

Lu, G., Villalba, M., Coscia, M. R., Hoffman, D. R. and King T. P. (1993). Sequence analysis and antigenic cross-reactivity of a venom allergen, antigen 5, from hornets, wasps, and yellow jackets. J Immunol 150, 2823-2830.

McKellar, Q. A. (1993). Interactions of *Ostertagia* species with their bovine and ovine hosts. Int J Parasitol 23, 451-462.

Moser, J. M., Freitas, T., Arasu, P. and Gibson, G. (2005). Gene expression profiles associated with the transition to parasitism in *Ancylostoma caninum* larvae. Mol Biochem Parasitol 143, 39-48.

Murray, J., Gregory, W. F., Gomez-Escobar, N., Atmadja, A. K. and Maizels, R. M. (2001). Expression and immune recognition of *Brugia malayi* VAL-1, a homologue of vespid venom allergens and *Ancylostoma* secreted proteins. Mol Biochem Parasitol 118, 89-96.

Nagasaki, H., Arita, M., Nishizawa, T., Suwa, M. and Gotoh, O. (2005). Species-specific variation of alternative splicing and transcriptional initiation in six eukaryotes. Gene. 30, 53-62. doi: 10.1016/j.gene.2005.07.027.

Parkinson, J., Mitreva, M., Whitton, C., Thomson, M., Daub, J., Martin, J., Schmid, R., Hall, N., Barrell, B., Waterston, R. H., McCarter, J. P. and Blaxter, M. L. (2004). A transcriptomic analysis of the phylum Nematoda. Nat Gen 36, 1259-1267.

Redmond, D. L., Smith, S. K., Halliday, A., Smith, W. D., Jackson, F., Knox, D. P. and Matthews, J.B. (2006). An immunogenic cathepsin F secreted by the parasitic stages of *Teladorsagia circumcincta*. Int J Parasitol 36, 277-286.

Schallig, H. D. F. H., Van Leeuwen, M. A. W., and Cornelissen A. W. C. A. (1997). Protective immunity induced by vaccination with two *Haemonchus contortus* excretory secretory proteins in sheep. Parasite Immunol 19, 447-453.

Sheth, N., Roca, X., Hastings, M. L., Roeder, T., Krainer, A. R. and Sachidanandam, R. (2006). Comprehensive splice-site analysis using comparative genomics. Nucleic Acids Res 34, 3955-3967.

Simpson, H. V., Simpson, B. H., Simcock, D. C., Reynolds, G. W. and Pomroy, W. E. (1999). Abomasal secretion in sheep receiving adult *Ostertagia circumcinta* that are prevented from contact with the mucosa. NZ Vet J 47, 20-24.

Simpson H. V. (2000). Pathophysiology of abomasal parasitism: is the host or parasite responsible? Vet J 160, 177-191.

Van Loon, L. C. and Van Strien, E. A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physio Mol Plant Pathol55, 85-97.

Van Zeveren, A. M., Visser, A., Hoorens, P. R., Vercruysse, J., Claerebout, E. and Geldhof, P. (2007). Evaluation of reference genes for quantitative real-time PCR in *Ostertagia ostertagi* by the coefficient of variation and geNorm approach. Mol Biochem Parasitol 153, 224-227.

Vercauteren, I., Geldhof, P., Peelaers, I., Claerebout, E., Berx, G. and Vercruysse, J. (2003). Identification of excretory-secretory products of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. Mol Biochem Parasitol 126, 201-208.

Chapter 4

Analysis of the transthyretin-like (TTL) gene family in *Ostertagia ostertagi* - comparison with other strongylid nematodes and *Caenorhabditis elegans*

*Based on the manuscript: Saverwyns, H., Visser, A., Van Durme, J., Power, D., Morgado, I., Kennedy, M.W., Knox, D.P., Schymkowitz, J., Rousseau, F., Gevaert, K., Vercruysse, J., Claerebout, E., Geldhof, P (2008). Analysis of the transthyretin-like (TTL) gene family in *Ostertagia ostertagi* - comparison with other strongylid nematodes and *Caenorhabditis elegans.* International Journal for Parasitology, accepted.

1. Introduction

As discussed in chapter 1, the use of excretory-secretory products (ESPs) as novel vaccine candidates and drug targets has been implicated in numerous studies (Knox, 2000; Geldhof *et al*., 2002; 2004; Meyvis *et al*., 2007). Recently, we studied another ESP, Oo-TTL-1, which belongs to the nematode-specific and conserved transthyretin-like (TTL) gene family, encoding proteins possessing a transthyretin-like domain (PF01060, IPR001534, DUF290). In mammalian parasites, TTLs have been detected in the ESPs from the cattle parasite *Ostertagia ostertagi* (Vercauteren *et al*., 2003), the human filarial nematode *Brugia malayi* (Hewitson *et al.,* 2008) and the sheep nematode *Haemonchus contortus* (Yatsuda *et al*., 2003). In the latter study, TTLs were considered to be the most immunogenic proteins based on Western blot analyses of ES products from adult worms probed with pooled sera from naturally immunized sheep. Furthermore, a recombinant form of a TTL of the dog hookworm Ancylostoma caninum has been evaluated as a vaccine candidate in dogs, in which a significant inverse correlation between antigen-specific IgE serum antibody titers and worm burden was observed (Hotez *et al*., 2003). TTLs were also identified in the free-living nematode *Caenorhabditis elegans* by Sonnhammer and Durbin (1997) and in the plant-parasitic nematodes *Xiphinema index* (Furlanetto *et al*., 2005), *Heterodera glycines* (Gao *et al*., 2003), *Meloidogyne incognita* (McCarter *et al*., 2003) and more recently in *Radophilis similis* (Jacob *et al.,* 2007).

As their name suggests, TTLs are a group of proteins with sequence similarity to the transthyretins (TTR) and transthyretin-related proteins (TRP). These three protein families are characterized by the presence of the transthyretin domain (PF00576). TTRs are specific to vertebrates and are present in plasma and extracellular fluids to transport thyroid hormones and, through their association with retinol binding protein, are also involved in the transport of retinol (vitamin A) (Sonnhammer and Durbin, 1997; Hennebry *et al*., 2006). On the other hand, TRPs are found in a much broader range of species, such as bacteria, plants, invertebrates and vertebrates, and are involved in a wide range of metabolic pathways (Lee *et al*., 2006; Li, 2005). In contrast, the role(s) of TTL proteins in nematodes is largely unknown. Based on their relationship with TTRs and TRPs, a number of functions have been postulated, including involvements in the transport of hormones (McElwee *et al*., 2004, Parkinson *et al*., 2004), digestion or absorption of nutrients (Furlanetto et al., 2005) and in the nervous system (Jacob et al., 2007).

The aim of the current study was (1) to investigate the TTL family in *O. ostertagi*, (2) to investigate the distribution of ttl genes and inferred proteins in *C. elegans* and selected parasitic nematodes of the order Strongylida and (3) to investigate their possible role(s) in the biology of nematodes.

2. Materials and methods

2.1. Parasite material

Infective larvae (L3) were obtained by culturing the faeces of calves infected with a laboratory strain of *O. ostertagi* (Geldhof *et al*., 2000). L4 and adult parasites were collected 10 and 21 days respectively after infection of nematode-free calves with respectively 500000 and 250000 L3 larvae of *O. ostertagi*. The L4 and adults were collected from the abomasum as previously described by Geldhof *et al.* (2000).

2.2. Bioinformatics

EST datasets for *O. ostertagi* were examined for the presence of ttl-sequences using the nematode-specific server NEMBASE2 (http://www.nematodes.org/nematodeESTs/nembase.html). Database searches for ttl homologues in the nematodes *C. elegans*, *Teladorsagia circumcincta*, *H. contortus*, *A. caninum*, *A. ceylanicum*, *Nippostrongylus brasiliensis* and *Necator americanus* were performed on NEMBASE2, Wormbase (www.wormbase.org) and NCBI server (http://www.ncbi.nlm.nih.gov/BLAST/). Alignment and analyses of these nucleotide and deduced amino acid sequences were conducted using the program DNASTAR (DNAstar Inc). Potential N-glycosylation sites were examined with PROSITE (www.expasy.ch/tools/scanprosite), whereas signal peptides were detected with SignalPV1.1 (www.cbs.dtu.dk/services/SignalP). Amino acid sequences were aligned using CLUSTALW (http://www.ebi.ac.uk/clustalw) and phylogenetic tree construction was carried out using of the program Mega v.3.1 (Kumar *et al*., 2004) by bootstrap test of phylogeny using the neighbour-joining algorithm and analyzing the consensus tree.

Secondary structures were predicted using the BioInfoBank Metaserver at http://meta.bioinfo.pl/submit_wizard.pl. The protein backbone from the C-alpha atoms was reconstructed using the SABBAC server (http://bioserver.rpbs.jussieu.fr.cgi-bin/SABAC) and the amino acid side chains were placed using FoldX (Schymkowitz *et al*., 2005). Subsequently, the structures were inferred using Yasara software (http://www.yasara.org).

2.3. Stage specific transcription

The levels of transcription of *Oo-ttl-1* in L3, exL3, L4 and adult *Ostertagia* were determined by reverse transcription-coupled real-time PCR (qPCR; q = quantitative) with the Lightcycler system using an LC-Fast Start reaction mixture with SYBR Green I (Roche Diagnostics). The RNA extraction and cDNA synthesis methods have been described previously (Van Zeveren *et al*., 2007). The qPCR reaction mixture (20 µl) consisted of a master mix with Taq DNA polymerase (ROCHE), dNTPs, SYBR green I, 3 mM MgCl2, 0.5 µM each of forward (Oo-TTLF2:

5'-TGATTCCGTCGTCGCAGTCATGG-3') and reverse (Oo-TTLR2: 5'-GGCGGCTGGAGTTACGGTGAAGC-3') primers and 2 µl of the template (cDNA or plasmid). The qPCR started with a denaturation step at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 64°C for 8 s and 72°C for 9 s. The qPCRs were performed in duplicate and a plasmid standard (representing the gene *Oo-ttl-1*) was included for quantification. The size and specificity of the PCR products was confirmed by agarose gel electrophoresis and melting curve analysis, respectively. To correct for sample-to-sample variation during the RNA isolation and reverse transcription steps, the raw data were normalised against the following house-keeping genes: β-actin (*Actb*), glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) and β-tubulin (*Tubb*). By means of the geNorm approach and the coefficient of variation, Van Zeveren *et al*. (2007) demonstrated that these 3 reference genes showed the highest transcriptional stability in the different *O. ostertagi* life stages. The relative amount of *Oo-ttl-1* mRNA transcription was plotted as a ratio ([number of copies of the *Oo-ttl-1* gene/normalisation factor based on *Actb/Gapdh/Tubb*]). This normalisation factor is basically the geometric mean of the transcription levels of these 3 reference genes

Another PCR approach was used to determine stage-and gender-specific transcription of other *ttl* contigs identified in the *O. ostertagi* EST dataset. The RNA extraction and cDNA synthesis have been previously described (Van Zeveren *et al*., 2007). The reaction mixture (12.5 µl) included 0.2 mM dNTPs, 1.5 mM MgCl2, 0.25 µM of both the forward and reverse, gene-specific primers, 1 U of Taq DNA polymerase (Invitrogen) and 0.5 µl of cDNA (1/10) prepared from the different life stages (L3, L4 and adults) of *O. ostertagi*. The various primer pairs used in this protocol can be found in table 4.1. The specificity of PCR amplification of each primer pair was confirmed by sequencing. Sequencing was carried out at the Center for Medical Genetics (University Hospital Ghent) by use of the dideoxy chain terminator method (Big Dye™ terminator v3.1, Applied Biosystems) and the ABI3730xl genetic analyzer (Applied Biosystems).

The PCR commenced with a denaturation at 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 10 min. To correct for sample-to-sample variation during RNA isolation and reverse transcription steps, the expression profile of the reference gene *rpl13* (structural component of 60S ribosomal subunit) was also included. The amplified products (150-250 bp) were resolved in agarose gels (2 %) and photographed using Quantity One 4.5.1 Chemidoc EQ™ Software System (Bio-Rad, CA, USA).

Table 4.1 The various primers used to determine stage and gender specific transcription of the 17 additional *Oo-ttl* genes.

Name (based on accessionnumber of contig)	DNA sequence (5' to 3')
OOC003459F	ACCTGATGGACGAGAAAACG
OOC003459R	TGCCATGGCTTACATAGCTG
OOC03170F	CGTCCGGAAAAACGAACTCT
OOC03170R	GGGCAGTTTGATGGTGAACT
OOC03116F	CGCCCAAAAAGGAATTTGAC
OOC03116R	GCAACTGCATTTGGTCCTTT
OOC00277F	AGGGAAACGACACAGAATGG
OOC00277R	GGAAGTTTCGCGTTCAAGTT
OOC00471F	GCCAACAATGTTCGAGTGAA
OOC00471R	AGGCTTAATGCCATCGTCAC
OOC03226F	CACTCGTGAATTTCGCTCAA
OOC03226R	AGCTTGAATTTGCCGTGACT
OOC01363F	GCGTCTGGGGTCTCTGTGAA
OOC01363R	ACACCTTGAAAACGGGATCA
OOC02081F	CTATACGGATGCCCATGGAT
OOC02081R	CCCCTCACCACCTGAAGTAA
OOC03147F	GAAACACACAGATGGCGTTG
OOC03147R	ATTGCTCTGGCTTCGGATTA
OOC03357F	GTGCGGTTATTCCGTGTCTA
OOC03357R	AAAACCATGTCGCAGTTGTG
OOC03629F	CAACTGGTAAGCCAGGGGTA
OOC03629R	CCAACGCCTACGTACTCCAT
OOC00657F	TTAGATCGCCCACAAAATCC
OOC00657R	AGAAAGCGGTAAGCGGTACA
OOC00166F	ACTCGCTGTCCTAGCTGCAT
OOC00166R	TCGCCTTCAGAGTTTGTGTG
OOC03545F	CGACTTGACCCCAATGATCT
OOC03545R	CCGAGACAATGTCCAAGGTT
OOC00565F	CAACGCAAGGTGTCGTTTTT
OOC00565R	CAAATGAGGCCAAGGTGTTT
OOC03759R	CCCCGCCTATCATTATCAGA
OOC00142F	TCCATGCAATACGAAACGAG
OOC00142R	GGTGTCCACGCGAAAATAGT

2.4. Recombinant expression of Oo-TTL-1

To clone the full-length *Oo-ttl-1*, total RNA from adult *O. ostertagi* was isolated using Trizol (Invitrogen) and subsequently converted into first-strand cDNA (Invitrogen). The full-length *Oo-ttl-1* cDNA sequence was obtained by PCR–based sequencing. In brief, the 3'-end was amplified using a gene-specific forward primer (TTL1F: 5'-GAATTCATGAAATATATAATTTTGATTGC-3') in combination with the oligo-dT primer. The 5'-end was PCR-amplified using a gene-specific reverse primer (TTLn3R: 5'-CCGTCGTCGCAGTCATGGTA-3') and the SL1 (5'-GGTTTAATTACCCAAGTTTGAG-3') primer. The amplicons were gel-

purified using a gel purification kit (NucleoSpinExtract II, Machery-Nagel) and then cloned in the pGEM-T Easy vector (Promega). Sequencing was performed as described in the previous section.

The full length sequence of *Oo-ttl-1* cDNA (414 bp) was cloned into the pGEMT-Easy vector (Promega Corporation) following PCR with primers, containing the restriction sites EcoRI and XhoI (Oottl1EcoRI: 5'-GAATTCATGAAATATATAATTTTGATTGC-3', Oottl1XhoI: 5'-CTCGAGAATCAATTCGCGTTCCTC-3'). The *Oo-ttl-1*-pGEM-T-Easy plasmid DNA was purified (using the Plasmid Midi kit, Qiagen) and cleaved with enzymes specific to the introduced restriction sites (Invitrogen). The digested *Oo-ttl-1* fragment was gel-purified and directionally ligated into the expression vector pASK-IBA43+. The ligation mixture was transformed into competent *Escherichia coli* BL21 (DE3)-codon plus (Invitrogen), and transformants were selected using the PCR employing the gene specific primers described above. The DNA sequence and open reading frame (ORF) were confirmed by sequencing. *E. coli* containing the *Oo-ttl-1*-pASK-IBA43+ construct was grown in 2 x YT broth at 37°C to an optical density (550 nm) of 0.6, after which recombinant Oo-TTL-1 protein (rOo-TTL-1) synthesis was induced by the addition of 0.2 µg/ml anhydrotetracycline for 2 h at 37°C. Cell lysates with the recombinant rOo-TTL-1 were prepared as described previously (Frangioni and Neel, 1993). Subsequently, rOo-TTL-1 was purified on a Strep-Tactin Sepharose column according to the manufacturer's protocol (Iba GmbH). The protein concentration and purity were estimated on SDS-PAGE followed by Coomassie blue staining by comparison with a bovine serum albumin standard (Pierce).

2.5. Production of monospecific antibodies

A laboratory rabbit was immunised three times subcutaneously with approximately 30 µg of purified recombinant Oo-TTL-1, at two week intervals. Each dose of antigen was supplemented with 5 µg Quil A (Superfos). Two weeks after the final immunisation, the antiserum was collected.

To reduce the potential for cross-reactions, the antiserum was affinity purified against rOo-TTL-1. Approximately 100 µg of rOo-TTL-1 was separated on SDS-PAGE and transferred to a polyvinyldine difluoride (PVDF) membrane (Immobilon®, Milipore). The Oo-TTL-1 region was excised and non specific binding was blocked with 10 % horse serum (HS) in PBST. The blot was incubated overnight at 4°C with the previously collected antiserum (1:40 diluted in PBST+ 2 % HS). This was followed by extensive washing with PBST the next day. Bound antibodies were eluted by incubating the blot with 5 ml of 5 mM glycine, 0.5 M NaCl, pH 2.5 for 5 min. The eluted antibodies were neutralised immediately with 100 µl 1M Tris pH 8.5. Finally HS (final concentration of 5 %) was added for stabilisation of the purified antibodies. These purified antibodies will be referred to as anti-Oo-TTL-1 serum (α-OoTTL-1).

2.6. SDS-PAGE and Western Blots

Somatic extracts (EX) from the L3, L4 and adult life stage were prepared as described by De Maere *et al.* (2002). ESPs from L3s, L4s and adults were collected as described by Geldhof *et al.* (2000). Ten micrograms of the *Ostertagia* EX and ESPs but also 1.5 µg of the positive control rOo-TTL-1 were separated on NuPAGE® 10 % Bis-Tris Gels (Invitrogen) under reducing and non reducing conditions and subsequently blotted on a PVDF membrane. Non specific binding was blocked by 10 % HS (in PBST). The blots were incubated with the α-OoTTL-1 serum overnight at 4°C. Subsequently, the blots were washed extensively with PBST (3 x 10 min) and incubated with goat anti-rabbit immunoglobulin (Sigma, 1:5000 in PBST with 2 % HS) conjugated with horse-radish peroxidase. 3.3 Diaminobenzidine tetrachloride in PBS and 0.02 % H_2O_2 were added to visualise the recognised proteins. An identical immunoblotting reaction was performed with 10 µg of *Cooperia oncophora* ES material.

To examine the recognition of Oo-TTL-1 during a natural infection, Western blots of rOo-TTL-1 (10 µg) were probed with pooled sera of animals naturally infected with *O. ostertagi* (1/ 200, 2 h at room temperature). Negative control sera were collected from the same animals at the beginning of the grazing season (pre-immune). After extensive washing with PBST, the blots were incubated with rabbit anti-bovine immunoglobulin (1/5000), conjugated with horse-radish peroxidase. 3.3 Diaminobenzidine tetrachloride in PBS and 0.02 % H_2O_2 were added to visualise the recognised proteins.

2.7. Immunolocalisation

Adult male and female *O. ostertagi* worms were collected separately, fixed for 24 hours in Bouin's solution (Sigma) and embedded in paraffin. Five µm-thick sections were made and paraffin was removed by xylene, isopropanol and a graded ethanol series (100%-70%). Sections were pretreated in a microwave with the Antigen Retrieval Citra Solution according to manufacturer's protocol (BioGenex). Sections were blocked with 20% goat serum for 30 minutes at room temperature and incubated with the monospecific α-OoTTL-1 (4 times concentrated) for 1 hour at 37°C. In the negative controls the α-OoTTL-1 serum was replaced by pre-immune rabbit serum (1:50 in 2% GS in PBS). Detection was carried out with Alexa Fluor 594 goat-α-rabbit IgG (Molecular Probes) (0,5mg/ml, 30min at 37°C). Red fluorescence was detected with a Leitz DMRB microscope (Leica instruments GmbH) by absorption of green light and pictures were taken using a Leica DC 100 camera (Leica instruments GmbH).

2.8. Hydrophobic ligand-binding and thyroid binding assay

The binding properties of recombinant Oo-TTL-1 were assessed in two different binding experiments. A spectrofluorometry-based binding analysis using 8-Anilino-1-naphthalenesulfonic acid (ANS) was used to probe non-specifically for hydrophobic regions of the protein in solution. ANS is a small, hydrophobic compound that has a low fluorescence emission in water, but emits at high levels when associated with exposed hydrophobic regions of misfolded proteins, or binding sites or pockets for hydrophobic ligands such as fatty acids or retinoids (McDermott *et al*., 2006).

Competitive binding studies with the purified recombinant protein using labelled thyroid hormones thyroxine [^{125}I]-T_4 and triiodothyronine [^{125}I]-T_3 were conducted as described by Morgado *et al.* (2007). Briefly, increasing concentrations of unlabeled T_4 an T_3 were added to TCN buffer (20mM Tris-HCl, pH 7.5, 93mM NaCl, 1mM $CaCl_2$ and 1mM $MgCl_2$) containing the rOo-TTL-1 and [^{125}I]-labelled thyroid hormones. rOo-TTL-1 bound T[^{125}I]-T_4 or [^{125}I]-T_3 were isolated and subsequently counted in a gamma counter (Wizard, Pharmacia-LKB).

2.9. PCR for assessing transcription and double-stranded RNA interference (RNAi) in *C. elegans*

The transcription of five *ttl* genes in *C. elegans* (JC8.8, JC8.14, T07C12.7, C56A3.2 and C14C10.7) was evaluated by reverse transcription PCR (One Step RT-PCR, Invitrogen). Different life stages (L1, L2, L3, L4 larvae and adult worms) of *C. elegans* were collected after synchronisation (Sulston and Hodgkin, 1988). RNA was prepared and used for the one step RT-PCR employing gene-specific primers (Geldhof *et al*., 2006). The various primer pairs used in this protocol can be found in table 4.2. To correct for sample-to-sample variation during RNA isolation and reverse transcription step, the transcription profile of the reference gene encoding *beta-tubulin* was also included as a control. The PCR products were resolved in agarose gels (2%) and photographed using the Quantity One 4.5.1 Chemidoc EQ™ Software System (Bio-Rad, CA, USA).

Table 4.2 The various primers used to evaluate the stage specific transcription of the 5 selected *Ce-ttls*.

name	DNA sequence (5' to 3')
T07C12.7F	TAAAGGGACGCCTTCTGTGT
T07C12.7R	CAAACGTTTTCTTGGCCACT
C56A3.2F	TCAGTCGATTGCTGTCAAGG
C56A3.2R	GGCCGGGAATGTACTTATCA
JC8.8F	TTGGCCATGAGACAACAG
JC8.8R	CAATTCACGTTCGGATCCTT
C14C10.7F	CCAATGTTCGCATAAAGCTG
C14C10.7R	ACACCTGCATCCATTGTTGA
JC8.14F	TTTGCTCTTTTGGTGCTCCT
JC8.14R	GAGTCCTGGCTTCAACTTGC

For the RNAi experiment, intron spanning fragments of these genes varying from 180 to 300 bp were cloned into the pGEM-T-Easy vector from mixed-stage cDNA (primer sequences can be found in table 4.2). Identity and specificity of the fragments was confirmed by sequencing. Subsequently the fragments were transferred to the L4440 RNAi vector using the NcoI and Sal I restriction enzymes. These L4440 constructs were linearised and dsRNA was produced from these templates using the T7 Ribomax Express RNAi system (Promega), as previously described by Geldhof *et al*. (2006). Previous RNAi experiments on the individual genes did not result in any visible phenotype, as reported on Wormbase (www.wormbase.org). For this reason, combinatorial RNAi, using the soaking protocol, was carried out to target the 5 *ttl* genes simultaneously. The 5 dsRNA samples were premixed with lipofectamin (Invitogen) and added to the culture medium with *C. elegans* L3s. After 24h of soaking, the larvae were transferred to NGM plates seeded with the OP50 *E. coli* stain (Geldhof *et al*, 2006). Subsequently, RNA of 20 adult hermaphrodites was prepared and the reduction of *ttl* transcripts was assessed using gene-specific primers (table 4.2) employing the Superscript One-Step RT-PCR system (Invitrogen), as recommended (35 cycles). Worms were visually inspected for general phenotypic effects like movement, growth and longevity. Also, a quantitative test was set up to assess reproduction and development. Twelve young hermaphrodites were plated individually on to NGM plates with the *E. coli* strain OP50 at 25 °C (Sulston and Hodgkin, 1988). Offspring produced over 24 h was counted as well as the number of adults after 32 h. For a heat-stress resistance assay, 10 young adult hermaphrodites were placed at 35°C for 2 h and scored every hour. The influence of *ttl* gene knock-down on fat storage was examined by feeding hermaphrodites with OP50 mixed with Nile Red, as described by Menzel *et al.* (2007).

3. Results

3.1. *O. ostertagi ttl* gene family

The first *ttl* sequence in *O. ostertagi* was identified by Vercauteren *et al*. (2003) following the immunoscreening of *Ostertagia* cDNA libraries with polyclonal rabbit serum raised against materials products from L3s, L4s or adults. These authors had isolated a partial *Oo-ttl-1* cDNA (AJ318795) sequence encoding a 125 amino acid (aa) protein with an incomplete N-terminus. In the present study, the missing 5'-end of the *Oo-ttl-1* gene was obtained from adult cDNA by PCR using the primer SL1 or oligo-dT in combination with gene-specific primers. The full length *Oo-ttl-1* cDNA sequence (414 bp; AM910982) was predicted to encode a protein of 138 aa with a mass of 15.3 kDa and a theoretical isoelectric point (pI) of 4.92. The predicted protein sequence contained a signal peptide with a consensus cleavage site between aa 15 and 16 (CLA-IR) (Fig. 4.1, marked with an arrowhead). Database searches with Oo-TTL-1 identified five TTL protein homologues, namely the proteins encoded by genes JC8.8 (e-value = 6e-44), C56A3.2 (e-value = 9e-45) and T07C12.7 (e-value = 3e-48) of *C. elegans*, the protein encoded by gene CBG19413 (e-value = 2e-49) of *C. briggsae* and the predicted protein Rs-TTL-4 (e-value = 4e-44) of *Rhadophilus similis* (see Fig. 4.1). All of the amino acid sequences contained two conserved cysteine residues and the characteristic 'TTL' domain with two conserved signature motifs (Fig. 4.1). All TTLs contained a predicted signal peptide with the exception of *R. similis* TTL-4, likely due to incomplete N-terminus of the predicted protein sequence of Rs-TTL-4 (cf. Jacob *et al*., 2007).

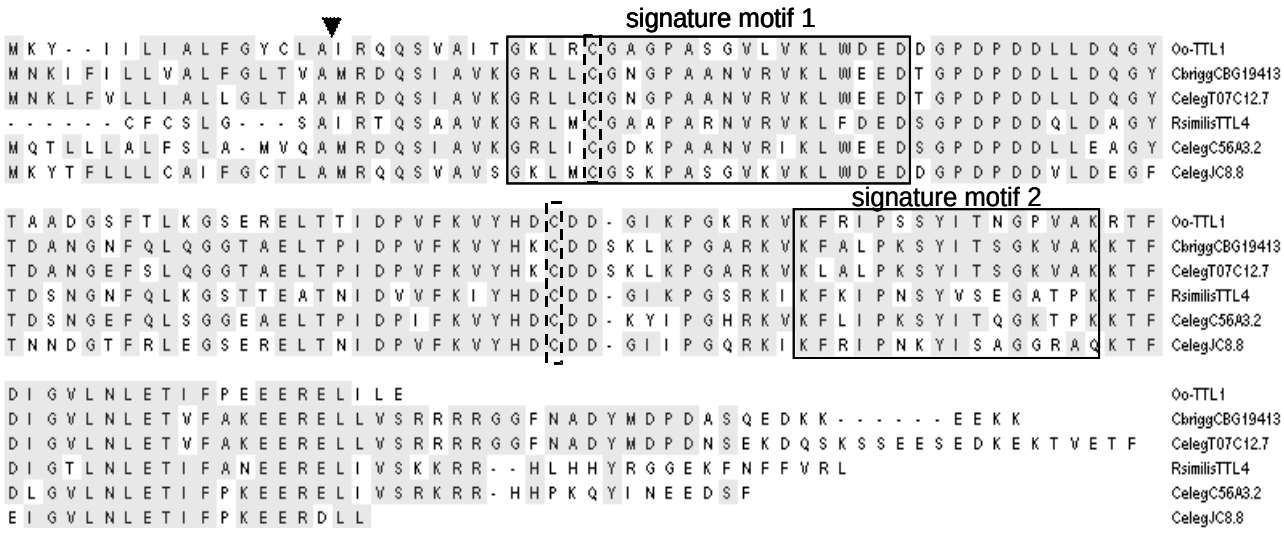


Figure 4.1 Alignment of the predicted amino acid sequences of Oo-TTL-1 and its closest homologues (*C. elegans* JC8.8= CAB05225, *C. elegans* T07C12.7= CAA98286, *C. elegans* C56A3.2= CAB01136, *C. briggsae* CBG19413= XP001674745, *R. similis* TTL-4= AM691120). The predicted cleavage site of the signal peptide of Oo-TTL-1 is indicated by an arrowhead. The 2 conserved signature motifs of the transthyretin like protein domain are boxed. Conserved cysteine residues are also indicated with a box.

The transcription of *Oo-ttl-1* was investigated in the different life cycle stages and both sexes by means of qPCR. The highest levels of transcription were detected in the adult male worms, whereas the levels in females and L4s were approximately two- and five-fold lower, respectively, and almost undetectable in L3s (Fig. 4.2).

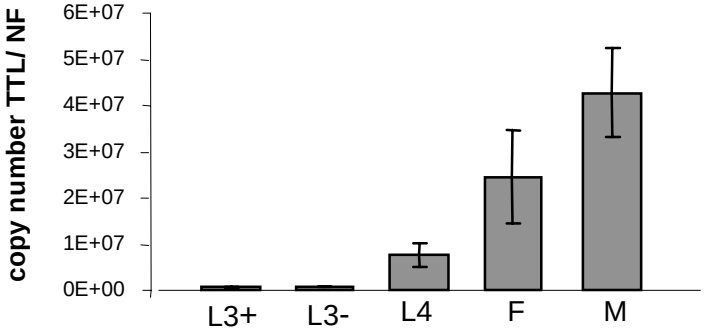


Figure 4.2 Stage- and gender- specific transcription of the *Oo-ttl-1* gene measured by quantitative PCR in the L3 stage with (L3+) and without sheath (L3-), the L4 stage, the adult females (F) and males (M). The relative amount of *Oo-ttl-1* mRNA transcription was plotted as a ratio ([number of copies of the *Oo-ttl-1* gene/normalisation factor based on Actb/Gapdh/Tubb]).

Affinity purified antibody against rOo-TTL-1 was used as probe in Western blot analysis of somatic and ES antigens/products from each of three parasitic life stages of *O. ostertagi*. The native protein (nOo-TTL-1) was most abundant in ESPs of adult parasites. A fainter band was detected in somatic products of the adult worms. The protein was not detected in the L3 and L4 stage (Fig. 4.3, panel A). Western blots of non-reduced adult ESPs, developed with the same antibodies suggested no dimerisation or tetramerisation under non-reducing conditions (results not shown). To investigate whether infected animals are exposed to Oo-TTL-1 or similar TTLs during the course of a natural infection, blots of the rOo-TTL-1 were probed with pooled sera of animals naturally infected with *O. ostertagi*. These sera detected a moderately strong band compared with the 'negative control' sera from pre-immune animals (Fig 4.3, panel B). The affinity purified anti-rOoTTL-1 antibody shown in Fig. 4.3 panel C, detected a band of ~ 15 kDa in ESPs from *Cooperia oncophora* (a small intestinal parasite of cattle).

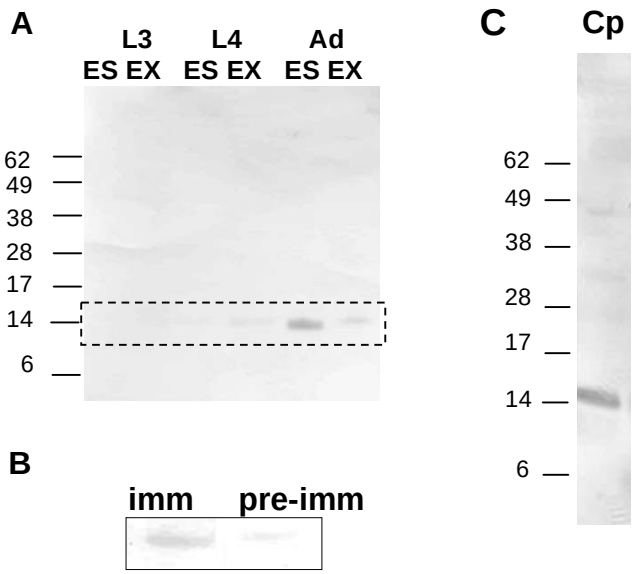


Figure 4.3 (A) Western blots of extracts (EX) and ESPs of the L3, L4 and adult life stage probed with monospecific anti-Oo-TTL-1 antiserum (under reducing conditions). (B) Recognition of rOoTTL-1 by pooled sera of pre-immune animals and animals naturally infected with *O. ostertagi*. (C) Western blots of *C. oncophora* (Co) ES material probed with monospecific anti-OoTTL-1 antiserum.

Native Oo-TTL-1 was immunolocalized in tissue sections from adult males or females of *O. ostertagi*. Specific red fluorescence was detected in pseudocoelomic fluid, with the strongest immunostaining located around the ovojector in females (Fig. 4.4, panel A) and the pharynx in males (Fig. 4.4, panel B). No staining was detected in sections of *O. ostertagi* probed with pre-immune rabbit serum or in the conjugate controls (data not shown).

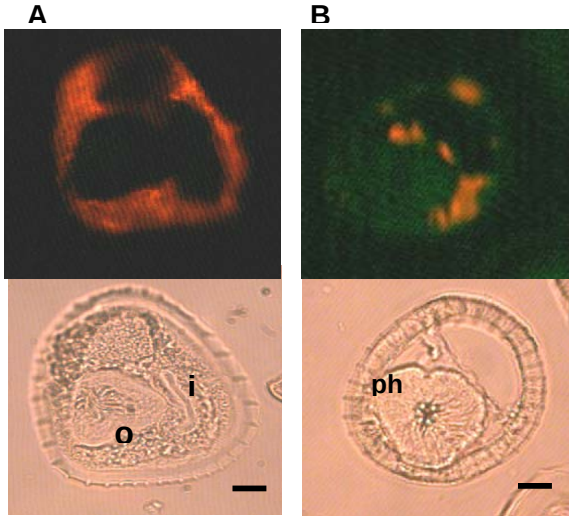


Figure 4.4 Immunolocalization of Oo-TTL-1 on a transverse section of an adult female (panel A) and male (panel B) parasite. (Bar= 25 µm)(o= ovojector, i= intestine, ph= pharynx)

Mining of EST data revealed the presence of 22 additional *ttl* contigs *in O. ostertagi.* Sequence analysis indicated that OOP03570 is possibly a splice variant of OOP03170. The predicted protein sequences of OOP03431 and OOP00093 were short and were identical to OOP03116 and Oo-TTL-1, respectively. Stage- and gender-specific transcription patterns of the 17 remaining *Oo-ttls* were determined by PCR. The results are shown in Fig. 4.5. Eleven *Oo-ttls* were constitutively transcribed throughout the life cycle of O. ostertagi, whereas two *Oo-ttls* were absent from the L4 stage (OOP00565, OOP00142). Two other *Oo-ttls* showed diminished transcription in the adult stage (OOP003759, OOP003116). Only one *ttl* (OOP001363) showed a similar transcription pattern to *Oo-ttl-1*, with transcription from the L4 stage onwards.

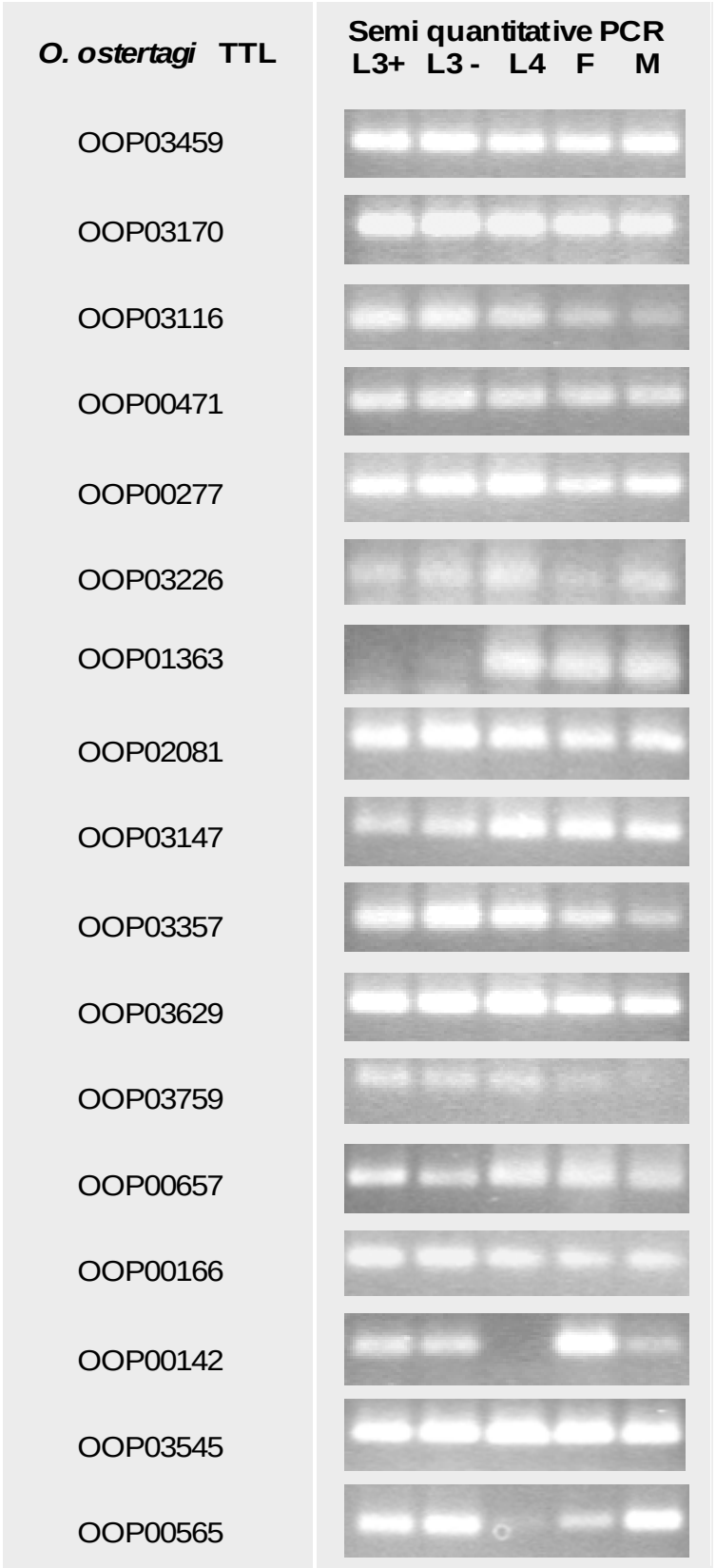


Figure 4.5 Stage and gender specific transcription of the other identified *ttl* contigs in the *O. ostertagi* EST dataset determined by RT-PCR in the L3 stage with (L3+) and without sheath (L3-), the L4 stage, the adult females (F) and males (M).

3.2. 3D modelling of Oo-TTL-1

In an attempt to model the three-dimensional structure of Oo-TTL-1, a BLAST search was conducted against the Protein Data Bank (PDB) to find a suitable template. Unfortunately, no sequences were found with an acceptable percentage of sequence similarity to our target sequence, indicating that conventional homology modelling was not feasible for Oo-TTL-1. Therefore, fold recognition methods were used to predict the overall fold of the Oo-TTL-1 sequence. The Oo-TTL-1 sequence was submitted to the 3D-jury server (Ginalski *et al.*, 2003), which returned the results from several secondary structure prediction servers using fold recognition. The results from four prediction servers indicated a beta-sheet rich structure (Fig. 4.6 panel A). According to this secondary structure profile, 3D-jury external modelling servers searched for structures in the PDB database resembling this particular structure prediction. The following criteria were used to judge the best model: The 3D-models from external modelling and prediction servers are processed by the 3D-Jury method. The models that are most similar to others have a higher chance to be correct and obtain higher 3D-Jury scores. The best scoring model came from HHPred2 (Söding *et al.*, 2005), probably because more residues were aligned than in models from other servers. This longer alignment might originate from the fact that HHPred2 returns multitemplate models, i.e. segments from multiple template structures are combined to assemble the model.

The template structures that were used by HHpred2 to build the Oo-TTL-1 model were the structures with PDB codes 2BOY, 1S9A, 1TMX, 2AZQ and 1DMH. Notwithstanding that the atomic details can never be reached using such fold recognition modelling methods, a full atomic model of Oo-TTL-1 was build to confirm and visualize the inside-outside (or core-surface) distribution of the residues located in the predicted beta-sheet regions. The 3D-jury analysis already provided atomic coordinates for the Oo-TTL-1 model from HHpred2, but only for the C-alpha atoms. The SABBAC server was subsequently used to reconstruct the full backbone of the model from the C-alpha coordinates. Finally, the FoldX software was applied to model the amino acid side chains on the Oo-TTL model. Upon visual inspection of the model, the residues located on the inside (buried part) of the beta sheets were found to constitute a hydrophobic core, with the exception of one buried histidine residue. Importantly, the low resolution of the Oo-TTL-1 model does not allow any precise structural analysis to atomic detail. However, the model does indicate that Oo-TTL-1 consists mainly of beta-sheets which are likely to be arranged in a beta-sandwich fold as shown in Fig. 4.6 panel B. Notably, a similar fold is also found in transthyretin monomers. We stress that this crude model should not be used for drug design or any other structure studies that require atomic precision. Our model is merely used to visualize the predicted beta-barrel fold structure of Oo-TTL-1.

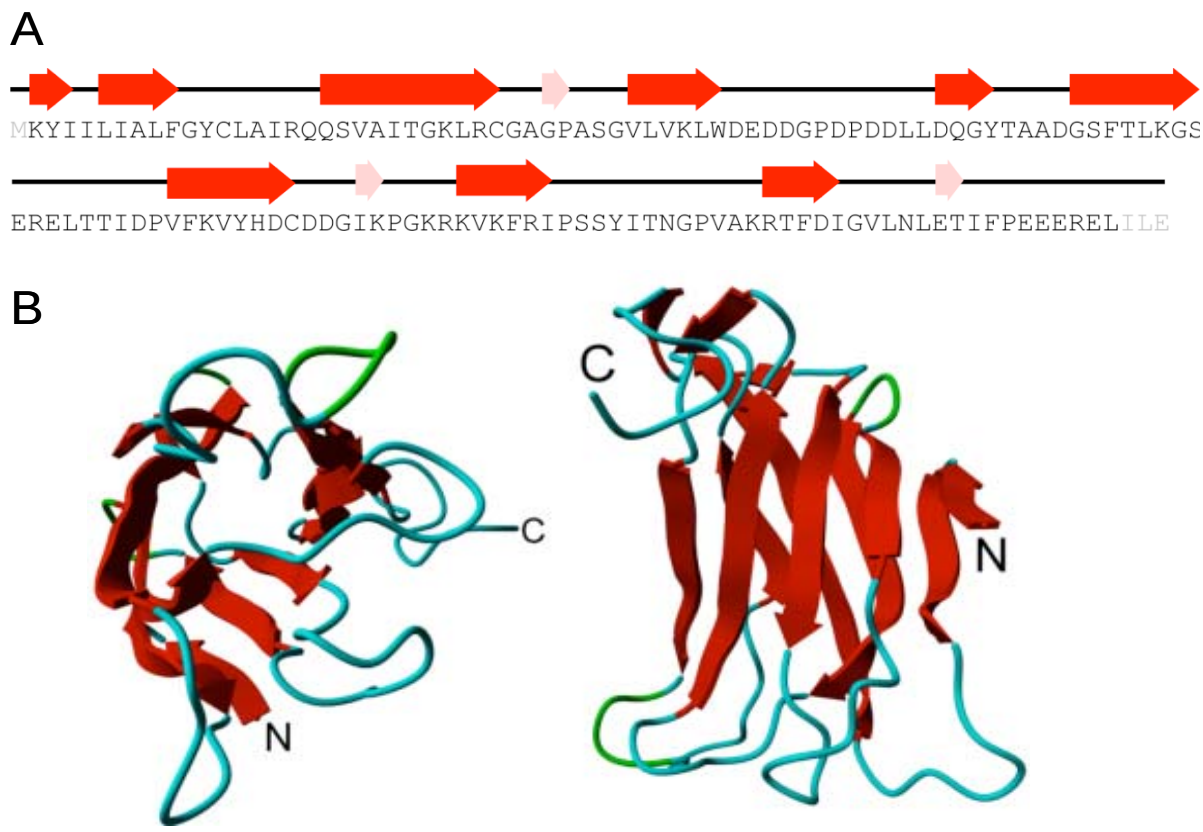


Figure 4.6 3D modelling of Oo-TTL-1. **(A)** 2D plot of secondary structure assignment in the Oo-TTL-1 model onto its sequence. Bright red arrows indicate beta-strands constituting the typical beta-sandwich fold. Light red arrows represent beta-sheet like regions in the outer loops. Greyed out residues were not modelled. **(B)** Alternative views of the Oo-TTL model where two layers of beta-sheets are arranged to form the beta-sandwich fold.

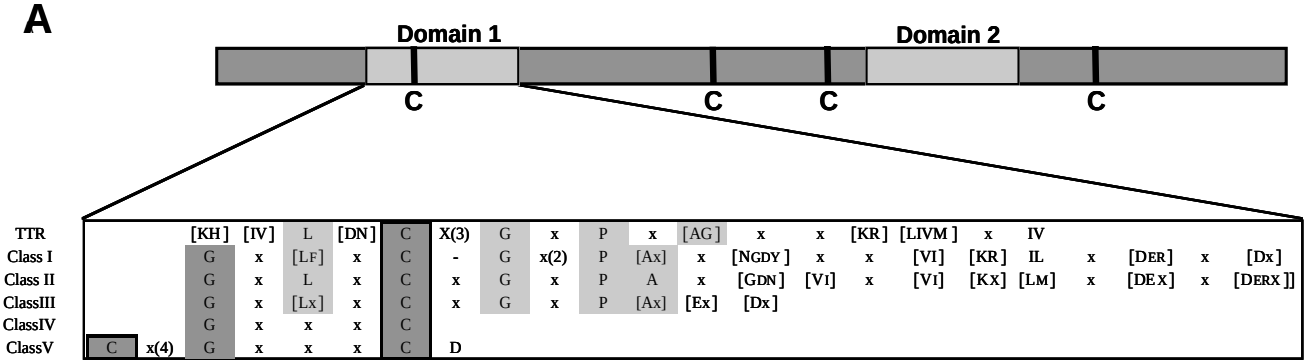
3.3. Thyroid, fat and retinol binding assay on Oo-TTL-1

To examine potential ligand binding properties, recombinant Oo-TTL-1 was first subjected to fluorescence-based ligand binding assays with ANS, a non-specific fluorescent probe for hydrophobic surfaces and binding pockets on proteins. Its fluorescence emission is enhanced upon entry into an apolar binding environment. Although a control fatty acid and retinol-binding protein (beta-lactoglobulin) demonstrated strong binding of ANS, the addition of rOo-TTL-1 failed to induce a significant increase in fluorescence intensity. These results suggest that recombinant Oo-TTL-1 is devoid of lipid binding properties. Moreover, unlike TTR tetramers that bind both T(4) and T(3), rOo-TTL-1 did not demonstrate any affinity for the thyroid hormones in the present study.

3.4. The *ttl* gene family for species of nematodes of the order Strongylida and *C. elegans*

In order to gain some insights on the presence of TTL proteins in other nematode species, the EST and sequence databases were screened *from C. elegans, T. circumcincta, H. contortus, A. caninum, A. ceylanicum, N. brasiliensis* and *N. americanus* for *ttl* sequences. In total, 185 *ttl* contigs were discovered: 56 in *C. elegans*, 18 in *T. circumcincta*, 43 in *H. contortus*, 21 in *A. caninum*, 29 in *A. ceylanicum*, 5 in *N. brasiliensis* and 13 in *N. americanus*. The corresponding protein sequences, including the 18 *O. ostertagi* TTLs, were subsequently submitted to a sequence analysis. A first alignment including all 185 sequences largely segregated the TTLs into 5 groups or classes. Sequence alignments of these different subfamilies were analyzed in detail to elucidate common and discriminating features and to define the different classes. The results of this analysis are shown in Fig. 4.7. TTR, TRP and TTL proteins are characterized by the presence of a similar protein domain, i.e. the transthyretin (PF00576) and the transthyretin-like (PF01060) protein domain. In the TTR protein domain 2 signature motifs are defined and the location of these domains in the TTLs is marked in Fig. 4.7 (panel A). The main criterion for subdividing TTL proteins was based on amino acid substitutions and level of conservation at the level of the first signature. The consensus sequences for each class are shown in Fig 4.7 (panel A). The sequences of the second signature motif were less conserved. In addition to the 2 motifs there is the presence of conserved cysteine residues. The majority of proteins were characterized by the presence of 4 cysteines. However, in class I and class IV respectively 48 and 5 proteins were found harbouring only the 2 first conserved cysteines (underlined in Fig. 4. 7 panel B). The classification of all TTL sequences of the nematode species included in this study is shown in Fig. 4. 7 panel B.

A



TTR			[KH]	[IV]	L	[DN]	C	X(3)	G	x	P	x	[AG]	x	x	[KR]	[LIVM]	x	IV						
Class I			G	x	[LF]	x	C	-	G	x(2)	P	[Ax]	x	[NGDY]	x	x	[VI]	[KR]	IL	x	[DER]	x	[Dx]		
Class II			G	x	L	x	C	x	G	x	P	A	x	[GDN]	[VI]	x	[VI]	[KX]	[LM]	x	[DEX]	x	[DERX]]		
ClassIII			G	x	[Lx]	x	C	x	G	x	P	[Ax]	[Ex]	[Dx]											
ClassIV			G	x	x	x	C																		
ClassV	C	x(4)	G	x	x	x	C	D																	

B

Class I	*O.ostertagia*	Oo-TTL1	OOP00471	OOP01363	OOP02081	OOP03116	OOP03147	OOP03357	OOP03629	OOP03759	
	T.circumcinta	TDP00163	TDP00171	TDP00185	TDP00254	TDP00420	TDP01119	TDP02265	TDP02325		
	H.contortus	HCP00365	HCP00705	HCP00756	HCP00760	HCP00775	HCP00830	HCP01741	HCP02240	HCP02251	HCP02564
		HCP02849	HCP03762	HCP05122	HCP10812	HCP10867	HCP12496				
	A.caninum	ACP00138	ACP00526	ACP00832	ACP00957	ACP01089					
	A.ceylaticum	AYP00274	AYP00281	AYP00386	AYP00418	AYP00432	AYP00516	AYP00517	AYP00594	AYP00608	AYP02925
		AYP02998	AYP03534								
	N.americanus	NAP00416	NAP00934	NAP01454	NAP01648						
	C.elegans	C14C10.7	C56A3.2	E02C12.13	E02C12.4	F10G7.11	F21E9.3	F22A3.2	F22A3.7	F58B3.9	H14N18.3
		JC8.8	JC8.14	T07C12.7	ZC64.2						
Class II	*O.ostertagia*	OOP00565	OOP03170	OOP03459	OOP03570						
	T.circumcinta	TDP02344									
	H.contortus	HCP00410	HCP00828	HCP02127	HCP02143	HCP02831	HCP06126	HCP06722			
	A.caninum	ACP00313	ACP00618	ACP00837	ACP01016	ACP01063	ACP04033				
	A.ceylaticum	AYP00374	AYP00505	AYP00609	AYP00878						
	N.americanus	NAP00457	NAP00636	NAP01550	NAP01617						
	C.elegans	B0334	C37C3.13	C40H1.5	F40F12.1	K03H1.3	K03H1.4	K03H1.6			
		R13A5.3	R13A5.6	T28B4.3	Y51A2D.9						
Class III	*O.ostertagia*	OOP00277	OOP03226								
	T.circumcinta	TDP00039	TDP00097	TDP00200	TDP00887						
	H.contortus	HCP00285	HCP00413	HCP00768	HCP00779	HCP01971	HCP02836				
	A.ceylaticum	AYP00417	AYP01305								
	C.elegans	C12D8.4	C33A12.15	F09F3.6	F19F10.4	F36A4.8	F46B3.3	F46B3.4	F56F4.2	F58A3.2	F58A3.5
		R90.2	R90.3	R90.4	T04H1.3	T08A9.2	T21C9.8	Y44A6B.4	Y51A2D.10	Y51A2D.11	
Class IV	*O.ostertagia*	OOP00142	OOP00657								
	H.contortus	HCP00831	HCP02771	HCP09417							
	N.americanus	NAP00756	NAP01787								
	C.elegans	F11F1.7	T05A10.3	T14G10.3	T14G10.4						
Class V	*O.ostertagia*	OOP00166	OOP03545								
	T.circumcinta	TDP00036	TDP00138								
	H.contortus	HCP00829	HCP00832	HCP01935	HCP06291						
	A.caninum	ACP00348	ACP03763								
	A.ceylaticum	AYP00326	AYP00461	AYP00627							
	N.brasiliensis	NBP00256	NBP00495	NBP00633	NBP00748	NBP00864					
	C.elegans	C04G2.1	F26G1.3	F26G1.10	F35G8.2	T07C4.5	T19C3.9	Y5F2A.1	Y5F2A.2		

Figure 4.7 (A) Schematic representation of the 5 classes of TTLs in Clade V nematoda. Conserved cysteine residues are marked by a black box. The 2 signature sequences similar to the transthyretin patterns are also indicated. The conserved amino acid sequence of the first TTL domain specific for each class is shown below. (B) Overview of the 5 groups of TTL proteins identified in *O. ostertagi, H. contortus, T. circumcincta, A. caninum, A. ceylanicum, N. brasiliensis, N. americanus* and *C. elegans.* TTLs with 2 conserved cysteine residues are underlined.

3.5. Stage specific transcription and RNAi in *Caenorhabditis elegans*

To elucidate a possible function for TTLs in nematode biology we performed RNAi in the free-living nematode *C. elegans*. In order to select suitable targets for the RNAi experiment, a multiple sequence alignment of Oo-TTL-1 and all *C. elegans* TTLs was done to determine conserved regions. Five Ce-TTLs (JC8.8, JC8.14, T07C12.7, C56A3.2 and C14C10.7) clustered with the *Ostertagia* Oo-TTL-1 sequence based on their sequence homology.

First, the stage specific transcription of the five selected *Ce-ttls* was evaluated by RT-PCR. The results are shown in Fig. 4.8. The transcription of JC8.14 and C56A3.2 was consistent throughout all life stages, with in the latter enrichment of transcription in L3 and adult worms. The genes T07C12.7 and JC8.8 showed a similar pattern where transcription was detected in L1s, L3s and mainly in adults. For C14C10.7, some transcription could only be detected in the adult worms.

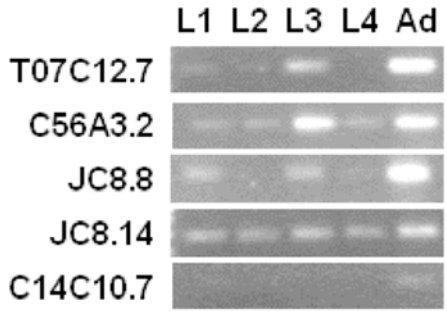


Figure 4.8 Stage specific transcription of the 5 selected *C. elegans ttl* sequences determined by RT-PCR in the L1, L2, L3, L4 and adult life stages.

Combinatorial RNAi on the five *C. elegans ttl* genes was performed to elucidate a possible function for the TTL protein family. The knock down/out was successful in 3 out of 5 Ce-*ttl* genes. These results are shown in Fig 4.9.

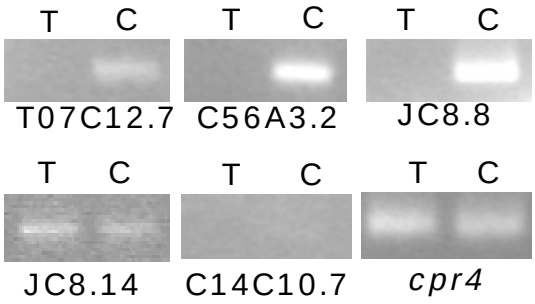


Figure 4.9 Combinatorial RNAi on the 5 selected *C. elegans ttl* genes and the reference gene *cpr4* (T= dsRNA-treated, C= control).

The transcription level of C14C10.7 was too low in the adult hermaphrodites to evaluate the success of the RNAi. On the other hand, the transcription of

JC8.14 could not be abrogated, not even when RNAi was performed solely for this gene (result not shown). No effect on movement, reproduction, development or longevity could be observed. Furthermore, no difference in fat content was detected nor were there differences in heat stress response (results not shown).

4. Discussion

Following an elaborate transcriptomic analysis of the phylum Nematoda, Parkinson *et al.* (2004) defined more than 4000 nematode-specific protein families encoded by nematode-restricted genes. One of the largest is the transthyretin like gene (TTL) family. In the present study, the full length cDNA of an *O. ostertagi ttl* gene (*Oo-ttl-1*) was obtained by a PCR approach and cloned for recombinant expression. Western blotting with specific antiserum showed that the native Oo-TTL-1 was mainly present in the ESPs of adult parasites. Furthermore, naturally infected animals raise an antibody response against Oo-TTL-1, indicating the exposure of these animals to Oo-TTL-1 or a related TTL under *in vivo* conditions. TTL proteins have also been detected in the ES of *C. oncophora*, *H. contortus* (Yatsuda *et al*., 2003) and *B. malayi* (Hewitson *et al*., 2008). Immunolocalisation of Oo-TTL-1 indicated the presence of this protein in the pseudocoelomic fluid of adult worms. These data seem to contradict the excretory-secretory character of Oo-TTL-1. However, TTLs may be released into the environment in a passive diffuse manner through the excretory system. The excretory canals of nematodes are exposed to the pseudocoelome and also connect to the hypodermis (Nelson *et al.,* 1983). So presumably substances may be transported in both directions for either waste removal into the channels or selective resorption of molecules from these channels. This has previously been observed for several lipid binding proteins (Solovyova *et al*., 2003; Jordanova *et al*., 2005).

A protein domain search using the NEMBASE database indicated that the TTL protein domain was represented 185 times in all nematodes studied, including 18 *ttl* genes in *O. ostertagi*. Most of these genes were constitutively transcribed in the parasitic life stages of *O. ostertagi*. A phylogenetic analysis on all TTL sequence data for all 7 species examined showed that they could be divided into at least 5 different classes of TTLs. Each class is characterized by specific amino acid sequences in the first TTL signature domain. For the parasitic species, most TTL sequences belonged to class I. In *H. contortus* and *A. ceylanicum*, respectively 16 (out of 43) and 12 (out of 29) TTL sequences belonged to this class, whereas in *C. elegans*, only 14 (out of the 56) TTLs of this class were present in the complete genome. This discrepancy might be the result of additional duplication events, which occurred during the evolution of the parasitic nematodes. It might also indicate the importance of the class I of TTLs in parasite biology. The largest number of TTLs in *C. elegans* belonged to class III, i.e. 19 TTL encoding genes. Interestingly, TTLs of class III were less represented

in the parasite EST datasets compared with those for *C. elegans*. The biological relevance of these different TTL classes remains unclear.

Based on the role of TTRs in vertebrates, various authors have suggested that TTLs may function as carriers of lipophilic substances or hormones (McElwee *et al*., 2004; Parkinson *et al*., 2004). Inspection of the theoretical 3D model for TTLs suggests that Oo-TTL-1 is composed of a similar beta-barrel fold as the previously experimentally determined model for TTRs (Lundberg *et al*., 2006). The TTR of humans needs to form tetramers, in order to create the ligand-binding site for thyroid hormones (Monaco *et al*., 1995; Naylor and Newcomer, 1999). This dimerisation and tetramerisation is dependent on the presence of a C-terminal beta-sheet in the TTR structure. Interestingly, this particular beta sheet was absent in the secondary structure prediction of Oo-TTL-1. The secondary structure prediction of the TTLs of *R. similis* showed that these proteins also possess the crucial beta-strands to form a similar beta-barrel fold to Oo-TTL-1, TTRs and TRPs (Jacob *et al*., 2007). However the particular beta-strand involved in dimerisation or tetramerisation is not present either, suggesting that these proteins do not form dimers. This seems to be, in agreement with the results of the Western blotting showing that under non-denaturing conditions, nOo-TTL-1 migrates at ~15 kDa, which is the size of a TTL monomer. Whereas under similar conditions, the dimer form of mammalian TTR are still clearly detectable (Episkopou *et al*., 1993). The binding studies with rOo-TTL-1 indicated that the protein was indeed devoid of any lipid or thyroid binding properties. It cannot be excluded that the bacterial recombinant was not folded as it would be in a eukaryotic system and therefore may not be active, so refolded proteins or alternative expression systems might be required. Nevertheless, such an assay has been shown previously to work using a bacterially produced TTR (Olofsson *et al*., 2001). Interestingly, the predicted sequence of TTL-4 of *R. similis* contains three additional predicted beta-strands in a basic C-terminal extension that is unique to this protein. However, there is no functional information available for this protein.

Jacob *et al.* (2007) suggested that, instead of transporting hormonal substances, the TTLs are likely to play a role in the nervous system of the nematodes. In *R. similis* the expression of *Rs-ttl-2* (class VI) was restricted to the ventral nerve. Similarly, the *ttl* gene R13A5.6 (Class IV) in *C. elegans* was found to be expressed in the nervous system and hypodermis. Also, the TTL family shows characteristics comparable with those of neuropeptides, i.e. a large protein family with secretion signals and different expression patterns between the members of the family. Moreover, Jacob *et al*. (2007) suggested that the lack of RNAi phenotypes for the majority of the ttl genes tested in *C. elegans* is in agreement with a neuronal expression pattern, since the neural system is recalcitrant to RNAi in *C. elegans*. Indeed, the genome wide RNAi screenings in *C. elegans* only reported aberrant phenotypes for 4 *ttl* genes (ttr-1, R13A5.3, T05A10.3, ZC64.2); these phenotypes included longevity, hyperactivity,

increased fat content, maternal sterility and larval death. No phenotypes were reported for the other 52 genes tested. In the present study, we targeted five *Ce-ttl* genes simultaneously. Although no phenotypes were detected, we were able to successfully knock down three of five *Ce-ttls* (based on transcriptional analysis by PCR), suggesting a non-neural localisation. The lack of a visible or detectable phenotype might be due to the functional redundancy within this large gene family. To investigate this further, we should consider combinatorial RNAi on a whole class of *ttl* genes. However, it should be noted that the lack of phenotype can also be attributed to an incomplete knock-out of the *Ce-ttls*, which cannot be confirmed by a non-quantitative RT-PCR. QPCR data of the RNAi experiments offers the solution and could provide conclusive information on the percentage of knock-down. In addition, more information on the transcription profile(s) and tissue distribution of the TTLs in *C. elegans* should be obtained in order to provide new insights in the biological role of the TTLs in nematodes.

5. References

Basavaraju, S., Zhan, B., Kennedy, M.W., Liu, Y., Hawdon, J. and Hotez, P.J. (2003). Ac-FAR-1, a 20 kDa fatty acid- and retinol-binding protein secreted by adult *Ancylostoma caninum* hookworms: gene transcription pattern, ligand binding properties and structural characterisation. Mol Biochem Parasitol 126, 63-71.

De Maere, V., Vercauteren, I., Saverwyns, H., Claerebout, E., Berx, G. and Vercruysse, J. (2002). Identification of potential protective antigens of *Ostertagia ostertagi* with local antibody probes. Parasitol 125, 383-391.

Episkopou, V., Maeda, S., Nishiguchi, S., Shimada, K., Gaitanaris, G., Gottesman, M.E. and Robertson, E.J. (1993). Disruption of the transthyretin gene results in mice with depressed levels of plasma retinol and thyroid hormone. Proc. Natl. Acad. Sci. USA 90, 2375-2379.

Frangioni, J.V. and Neel, B.G. (1993). Solubilization and purification of enzymatically active glutathione S-transferase (pGEX) fusion proteins. An Biochem 210, 179-187.

Furlanetto, C., Cardle, L., Brown, D.J.F. and Jones, J.T. (2005). Analysis of expressed sequence tags from the ectoparasitic nematode *Xiphinema index*. Nematod 7, 95- 104.

Gao, B., Allen, R., Maier, T., Davis, E.L., Baum, T.J. and Hussey, R.S. (2003). The parasitome of the phytonematode *Heterodera glycines*. Mol Plant Microbe Interact 16, 720-6.

Garofalo, A., Rowlinson, M.C., Amambua, N.A., Hughes, J.M., Kelly, S.M., Price, N.C., Cooper, A., Watson, D.G., Kennedy, M.W. and Bradley, J.E. (2003). The FAR protein family of the nematode *Caenorhabditis elegans*. Differential lipid binding properties, structural characteristics, and developmental regulation. J Biol Chem 278, 8065-74.

Geldhof, P., Claerebout E., Knox, D.P., Agneessens J. and Vercruysse, J. (2000). Proteinases released in vitro by the parasitic stages of the bovine abomasal nematode *Ostertagia ostertagi*. Parasitol 121, 639-647.

Geldhof, P., Claerebout, E., Knox, D., Vercauteren, I., Looszova, A. and Vercruysse, J. (2002). Vaccination of calves against *Ostertagia ostertagi* with cysteine proteinase enriched protein fractions. Parasite Immunol 24, 263-270.

Geldhof, P., Vercauteren, I., Vercruysse, J., Knox, D.P., van den Broeck, W. and Claerebout, E. (2004). Validation of the protective *Ostertagia ostertagi* ES-thiol antigens with different adjuvantia. Parasite Immunol 26, 37-43.

Geldhof, P., Molley, C. and Knox, D. (2006). Combinatorial RNAi on intestinal cathepsin B-like proteinases in *Caenorhabditis elegans* questions the perception of their role in nematode biology. Mol biochem Parasitol 145, 128-132.

Geldhof, P., Visser, A., Clark, D., Saunders, G., Britton, C., Gilleard, J., Berriman, M. and Knox, D. (2007). RNA interference in parasitic helminths: current situation, potential

pitfalls and future prospects. Parasitol 134, 609-19.

Ginalski, K., Elofsson, A., Fischer, D.and Rychlewski, L. (2003) 3D-Jury: a simple approach to improve protein structure predictions. Bioinf 19, 1015-1018.

Hennebry, S.C., Wright, H.M., Likic, V.A. and Richardson, S.J. (2006). Structural and functional evolution of transthyretin and transthyretin-like proteins. Prot 64, 1024-45.

Hewitson, J.P., Harcus, Y.M., Curwen, R.S., Dowle, A.A., Atmadja, A.K., Ashton, P.D., Wilson, A. and Maizels, R.M. (2008). The Secretome of the Filarial Parasite, *Brugia malayi*: Proteomic Profile of Adult Excretory-Secretory Products. Mol Biochem Parasitol, in press.

Hotez, P.J., Zhan, B., Bethony, J.M., Loukas, A., Williamson, A., Goud, G.N., Hawdon, J.M., Dobardzic, A., Dobardzic, R., Ghosh, K., Bottazzi, M.E., Mendez, S., Zook, B., Wang, Y., Liu, S., Essiet-Gibson, I., Chung-Debose, S., Xiao, S., Knox, D., Meagher, M., Inan, M., Correa-Oliveira, R., Vilk, P., Shepherd, H.R., Brandt, W. and Russell, P.K. (2003). Progress in the development of a recombinant vaccine for human hookworm disease: The Human Hookworm Vaccine Initiative. Int J Parasitol 33, 1245-58.

Olofsson, A., Ippel, H.J., Baranov, V., Hörstedt, P., Wijmenga, S. and Lundgren, E.J. (2001). Capture of a dimeric intermediate during transthyretin amyloid formation. Biol Chem 276, 39592-9.

Jacob, J., Vanholme, B., Haegeman, A. and Gheysen, G. (2007). Four transthyretin-like genes of the migratory plant-parasitic nematode *Radopholus similis*: members of an extensive nematode-specific family. Gene 402, 9-19.

Jordanova, R., Radoslavov, G., Fischer, P., Liebau, E., Walter, R.D., Bankov, I. and Boteva, R. (2005). Conformational and functional analysis of the lipid binding protein Ag-NPA-1 from the parasitic nematode *Ascaridia galli*. FEBS J 272, 180-9.

Klesius, P.H. (1993). Regulation of immunity to *Ostertagia ostertagi*. Vet Parasitol 46, 63-79.

Knox, D.P. (2000). Development of vaccines against gastrointestinal nematodes. Parasitol 120, S43-S61.

Kumar, S., Tamura, K. and Nei, M. (2004). MEGA3: Integrated Software for Molecular Evolutionary Genetics Analysis and Sequence Alignment. Brief Bioinf 5, 150-163.

Lizotte-Waniewski, M., Tawe, W., Guiliano, D.B., Lu, W., Liu, J., Williams, S.A. and Lustigman, S. (2000). Identification of potential vaccine and drug target candidates by expressed sequence tag analysis and immunoscreening of *Onchocerca volvulus* larval cDNA libraries. Infect Immun 68, 3491-501.

Lee, Y., Park, B.C., Lee, do. H., Bae, K.H., Cho, S., Lee, C.H., Lee, J.S., Myung, P.K. and Park, S.G. (2006). Mouse transthyretin-related protein is a hydrolase which degrades 5-hydroxyisourate, the end product of the uricase reaction. Mol Cells 22, 141-5.

Li, J. (2005). Brassinosteroid signalling: from receptor kinases to transcription factors. Curr Opin Plant Biol 8, 526-531.

Lundberg, E., Bäckström, S., Sauer, U.H. and Sauer-Eriksson, A.E. (2006). The transthyretin-related protein: structural investigation of a novel protein family. J Struct Biol 155, 445-57.

McCarter, J.P., Mitreva, M.D., Martin, J., Dante, M., Wylie, T., Rao, U., Pape, D., Bowers, Y., Theising, B., Murphy, C.V., Kloek, A.P., Chiapelli, B.J., Clifton, S.W., Bird, D.M. and Waterston, R.H. (2004). Analysis and functional classification of transcripts from the nematode *Meloidogyne incognita*. Genome Biol 4, R26.

McDermott, L.C, Freel, J.A., West, A.P., Bjorkman, P.J. and Kennedy, M.W. (2006). Zn-α_2-glycoprotein, an MHC class I-related glycoprotein regulator of adipose tissues: Modification or abrogation of ligand binding by site-directed mutagenesis. Biochem 45, 2035-2041.

McElwee, J.J., Schuster, E., Blanc, E., Thomas, J.H. and Gems, D. (2004). Shared transcriptional signature in *Caenorhabditis elegans* Dauer larvae and long-lived daf-2 mutants implicates detoxification system in longevity assurance. J Biol. Chem 279, 44533-43.

Menzel, R., Yeo, H.L., Rienau, S., Li, S., Steinberg, C.E. and Stürzenbaum, S.R. (2007). Cytochrome P450s and short-chain dehydrogenases mediate the toxicogenomic response of PCB52 in the nematode *Caenorhabditis elegans*. Mol Biol 370, 1-13.

Monaco, H.L., Rizzi, M. and Coda, A. (1995) Structure of a complex of two plasma proteins: transthyretin and retinol-binding protein. Sci 268,1039- 1041.

Morgado, I., Hamers, T., Van der Ven, L. and Power, D.M. (2007). Disruption of thyroid hormone binding to *sea bream* recombinant transthyretin by ioxinyl and polybrominated diphenyl ethers. Chemosphere 69, 155-63.

Meyvis, Y., Geldhof, P., Gevaert, K., Timmerman E., Vercruysse J. and Claerebout E. (2007). Vaccination against *Ostertagia ostertagi* with subfractions of the protective ES-thiol fraction. Vet Parasitol 149, 239-45.

Naylor, H.N. and Newcomer, E. (1999). The structure of human retinol-binding protein (RBP) with its carrier protein transthyretin reveals an interaction with the carboxy terminus of RBP. Biochem 38, 2647-2653.

Nelson, F.K., Albert, P.S. and Riddle, D.L. (1983). Fine structure of *the Caenorhabditis elegans* secretory-excretory system. J Ultrastruct Res 82, 156-71.

Olofsson, A., Ippel, H.J., Baranov, V., Hörstedt, P., Wijmenga, S. and Lundgren, E.J. (2001). Capture of a dimeric intermediate during transthyretin amyloid formation. Biol. Chem. 276, 39592-9.

Parkinson, J., Mitreva, M., Whitton, C., Thomson, M., Daub, J., Martin, J., Schmid, R., Hall, N., Barrell, B., Waterston, R.H., McCarter, J.P. and Blaxter, M.L. (2004). A

transcriptomic analysis of the phylum Nematoda. Nat Genet 36,1259-67.

Schymkowitz, J., Borg, J., Stricher, F., Nys, R., Rousseau, F. and Serrano, L. (2005). The FoldX web server: an online force field. Nucl Acids Res 33, W382-W388.

Söding, J., Biegert, A. and Lupas, A.N. (2005). The HHpred interactive server for protein homology detection and structure prediction. Nucl Acids Res. 33 (Web Server issue), W244-8.

Solovyova, A.S., Meenan, N., McDermott, L., Garofalo, A., Bradley, J.E., Kennedy, M.W. and Byron, O. (2003). The polyprotein and FAR lipid binding proteins of nematodes: shape and monomer/dimer states in ligand-free and bound forms. Eur Biophys J. 32, 465-76.

Sonnhammer, E.L. and Durbin, R. (1997). Analysis of protein domain families in *Caenorhabditis elegans*. Genomics 46, 200-16.

Sulston,J. and Hodgkin,J. (1988). Methods. In Wood,W.B. (ed.), The Nematode *Caenorhabditis elegans.* Cold Spring Harbor Laboratory Press, New York, NY, pp. 587-606.

Van Zeveren, A.M., Visser, A., Hoorens, P.R., Vercruysse, J., Claerebout, E. and Geldhof, P. (2007). Evaluation of reference genes for quantitative real-time PCR in *Ostertagia ostertagi* by the coefficient of variation and geNorm approach. Mol. Biochem Parasitol 153, 224-227.

Vercauteren, I., Geldhof, P., Peelaers, I., Claerebout, E., Berx, G. and Vercruysse, J. (2003). Identification of excretory-secretory products of larval and adult *Ostertagia ostertagi* by immunoscreening of cDNA libraries. Mol Biochem Parasitol 126, 201-208.

Visser, A., Geldhof, P., de Maere, V., Knox, D.P., Vercruysse, J. and Claerebout, E. (2006). Efficacy and specificity of RNA interference in larval life-stages of *Ostertagia ostertagi*. Parasitol 133, 777-83.

Yatsuda, A.P., Krijgsveld, J., Cornelissen, A.W., Heck, A.J. and de Vries, E. (2003). Comprehensive analysis of the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition. J Biol Chem 278, 16941-51.

Chapter 5

General discussion and conclusion

1. Introduction

The objective of this project was to characterise *O. ostertagi* ESPs and to study their interaction with bovine abomasal proteins. In Chapter 2 we assessed the use of the phage display technique for the discovery and evaluation of the interactions of ES molecules with the host. The versatile phage display technology has made significant contributions to parasitology research, especially for analyzing molecular interactions between the parasite and its host (Jefferies, 1998; Viaene *et al*., 2001; Gnanasekar *et al*., 2004; 2005; Lanzillotti and Coetzer, 2007) and for aiding in the identification of epitopes and antibodies with potentially improved diagnostic and therapeutic value (Zhang *et al*., 2007). However, we demonstrated that the cDNA phage display technology clearly has its limitations when studying protein-protein interactions. Furthermore, it should be emphasized that cDNA phage display is just one of many molecular tools to study protein-protein interactions. Therefore, in the next paragraphs we will outline alternative tools, which have been used in the past to study protein-protein interactions.

Although we were unable to identify biologically relevant protein-protein interactions with the phage display technology, the importance of ESPs in the infection process remains unquestionable. Hence, in chapters 3 and 4 we characterised several novel ES components and their corresponding nematode specific protein families (the ASP-like and Transthyretin-like protein families). In the following paragraphs, we will further discuss the putative function of these nematode specific protein families. Finally, some concluding remarks are summarised in the last paragraph.

2. Tools to study the host-parasite interaction

Due to the growing interest in protein–ligand interactions, new technologies and approaches for studying the host–parasite interactions are still emerging. Over the last decades, numerous results obtained by phage display have yielded insight into parasitic diseases (Lanzilotti and Croetzer, 2008; Yao *et al*., 2007; Lauterbach *et al*., 2003; Viaene *et al*., 2001; Jespers *et al*., 1995). It remains without doubt that this technology is an extremely powerful and versatile tool that has multiple applications in parasitology research, especially for probing molecular interactions between the parasite and the host (Lanzillotti and Croetzer, 2007). Moreover, the technique is simple, cheap, rapid to set up and requires no special equipment (Willats, 2002). In this thesis, we utilized the filamentous M13 pVI phage display method (Jespers *et al*., 1995). The filamentous phage is an excellent cloning vehicle because insertion of foreign sequences within their genome results in assembly of longer phage particles (Willats, 2002). Furthermore, to overcome the limitations of direct fusions of cDNA to the N-terminus of phage coat proteins pIII or pVIII due to translational stop codons present in the 3’ end of the cDNA, we fused abomasal and *O.*

ostertagi cDNA libraries to the C-terminus of the minor phage coat protein pVI (Jespers *et al*., 1995). However, a pivotal drawback of using filamentous phage display remains the non-lytic propagation mechanism of this phage that requires export of all the phage coat components through the bacterial inner membrane before phage assembly (Hufton *et al.*, 1999; Danner and Belasco, 2001). Consequently, only a subset of proteins encoded by a given cDNA library will be capable of display on M13 phage. This ability for export to the surface depends on the length, sequence and folding characteristics of each protein to be displayed (Danner and Belasco, 2001).

In principle, this limitation may be avoided by using the lytic phages Lambda and T7, in which capsid assembly occurs entirely in the cytoplasm prior to cell lysis (Ansuini *et al*., 2002; Castillo *et al*., 2001; Cicchini *et al*., 2002; Clark and March, 2004; Yao *et al.,* 2007; Bair *et al*., 2008). Recently, Yao *et al*. (2007) successfully developed a T7 phage display screening method based on *Cryptosporidium parvum* cDNA in order to identify a new surface adhesion protein on sporozoite and oocyst. Furthermore, recent studies have shown that unlike T7, Lambda phage can tolerate the display of relative large proteins at high density (Zucconi *et al*., 2001; Gupta *et al*., 2003). Lambda proteins gpD and gpV are both excellent platforms for display (Hoess, 2002). A novel lambda gpD vector was designed to highly enrich for clones containing open reading frames (ORFs). *In vivo* biotinylation of the fusion phage during amplification followed by streptavidin affinity chromatography resulted in a higher percentage of clones with the correct ORF and a low background of false positive clones (Ansuini *et al*., 2002). Additionally, Castillo *et al*. (2001) combined different phage types in one experiment: whilst the peptide targets were displayed on T7, the anti-peptide single chain antibody fragments (scFvs) were selected from an M13 display library. To conclude, each of these phage display (M13, T7, Lambda) methods has its own advantages and disadvantages with respect to each particular application.

As mentioned in chapter 2, transmembrane proteins and receptor molecules appear to be problematic for traditional phage display. A more suitable method could be **the MAPPIT technique** (the Mammalian Protein-Protein Interaction Trap) that was originally designed to study known ligand-surface receptor interactions in order to identify novel targets for therapy in allergic disease (reviewed by Tavernier et al., 2002). Furthermore, the interactions between prey and bait occur in the cytosolic, submembrane space of the mammalian cell. Alternatively, genomics have also proven to be an infinite resource of information to study the host-parasite homeostasis. During an infection, the host-parasite interface is controlled by many genes from both the host and parasite genomes (Yang et al., 2008). Hence, identification of differentially regulated genes in host and parasite at a particular point during infection may facilitate the discovery of new important host-parasite interactions. Several genomic tools for the identification of those differentially regulated genes include the differential

display technique (Cui *et al*., 2001; Ghosh *et al*., 2003), subtractive cDNA hybridisation (Nisbet et al., 2008) and the microarray technology (Moser *et al*., 2005; reviewed by Caillaud *et al*., 2008). Additionally, proteomics has resulted in the identification of ESPs that are differentially expressed at a particular time in the parasite's life cycle or between different species (Yatsuda *et al*., 2003; Craig *et al*., 2006; Robinson *et al*., 2007). Hence, proteomics could aid to identify key parasite and host proteins that are involved in the host-parasite interaction.

3. Nematode specific protein families

Increasing incidence of anthelmintic resistance in parasitic nematodes has resulted in the extensive search for appropriate vaccine and drug candidates. These target genes for chemotherapy and vaccination ideally have to meet certain criteria: they should be nematode-specific, conserved and essential for the parasite's survival and/or development (Lizotte-Waniewski *et al*., 2000; Jacob *et al*., 2007). These requirements should assure minimal side effects in the host in combination with a broad activity against nematode parasites of medical and veterinary importance. In the search of such gene families, Parkinson *et al*. (2004) initiated two sequencing projects that generated more than 265,000 expressed sequence tag (EST) sequences from 30 species across the phylum Nematoda. The analysis identified hundreds of nematode-specific and conserved gene families. Two such nematode-specific and secreted protein families were investigated in this thesis.

3.1. ASP-like proteins

In chapter 3, we analysed the ASP-like (AL) protein family, a novel specific secreted protein family for selected members of the subfamily Ostertagiinae (Nematoda). The AL protein family contains the SCP/TPX-1/Ag5/PR-1/Sc7 protein domain which is also present in the activation associated secreted protein family of nematodes (ASPs). Nevertheless, sequence analysis indicated that the AL proteins are distinct from all the previously identified ASPs. As described in chapter 3, the precise function of the nematode ASPs and ASP-like proteins remains unknown. Many authors have suggested that ASPs take part in the infection process and the transition to parasitism, referring to their abundance in ESPs of L3 worms. (Hawdon *et al*., 1996, 1999; Murray *et al*., 2001; Moser *et al*., 2005). However, the AL genes of *Ostertagia* and *Teladorsagia* were predominantly transcribed in the adult and L4 life stages, respectively. Furthermore, the Oo-AL protein was almost exclusively detected in the adult ESPs.

A recent study by Visser *et al.* (2008) sheds another light on the functional role of ASP proteins. They observed a male enriched transcription pattern in 9 out of 15 *O. ostertagi* ASP genes, which might indicate a role in reproduction or

in the development of the reproductive system. Similar observations were made in *Caenorhabditis elegans*, *Trichostrongylus vitrinus*, *Oesophagostomum dentatum* and *Brugia malayi* (Reinke *et al*., 2004; Nisbet and Gasser, 2004; Li *et al.*, 2005; Cottee *et al*., 2006). Furthermore, other members of the SCP/TPX-1/Ag5/PR-1/Sc7 protein family have been appointed to male-enriched clusters. For example, major sperm proteins were easily retrieved from male-enriched microarray datasets of *B. malayi*, *T. vitrinus* and *C. elegans* (Li *et al.*, 2005; Reinke *et al*., 2000). However, this reproductive function cannot be generalised for all members of the SCP/Tpx-1/Ag5/PR-1/Sc7 family. Mouse AEG2 is secreted in the salivary glands, despite its sex-specific transcription and expression (Mizuki and Kasahara, 1992). Also, *O. ostertagi* Oo24 was immunolocalized in the pharynx, which seems to contradict a role in parasite reproduction (Visser *et al*., 2008). The male enrichment on transcriptional level of many of the ASPs in *O. ostertagi* might actually be a remnant of an ancestral ASP molecule that might have had an important function in male reproduction.

In conclusion, further unraveling the functional role of the AL proteins will be a difficult task. The tools to perform functional genomics in parasitic nematodes are very limited. Although RNA interference is widely used in *C. elegans*, the utility of this technology in *O. ostertagi* is still questionable (Visser *et al.*, 2006; Geldhof *et al*., 2007). For this reason, *C. elegans* has been a popular model organism for functional research on nematode specific genes. Unfortunately, in the case of the AL proteins, it is of no use since *C. elegans* does not have any homologues.

3.2. Transthyretin-like proteins

In chapter 4 we have attempted to unravel the composition, distribution and biology of the Transthyretin-like (TTL) protein family in Clade V nematodes. We have confirmed their widespread occurrence in Clade V and were able to subdivide this elaborate nematode specific protein family into seven classes. However, up till now the function of TTL proteins remains obscure. To investigate a possible gene function of nematode TTLs, several experimental binding assays and combinatorial RNAi were performed but no conclusive information on their functionality was obtained.

According to Jacob *et al*. (2007), database screens of translated nematode ESTs indicated that the fraction of *ttl*-ESTs was larger in parasitic nematodes compared to free-living nematodes with a bias for libraries constructed from the parasitic stages, suggesting a possible role in host-parasite interactions. However, for the majority of the *Oo-ttls*, transcription was detected from the non-parasitic L3 life stage onwards. This constitutive transcription pattern seems contradictory to a possible role in infection and transition to parasitism.

Furthermore, combinatorial RNAi with the 5 Class I *C. elegans ttls* did not evoke a detectable phenotype. Nevertheless, knocking out combinations of genes can have profoundly different effects to that of knocking out each gene individually (Fortunato and Fraser, 2005). Therefore, as suggested in chapter 4, one could still simultaneously target all the members of the remaining classes or even target the *C. elegans ttl* gene family in total to exclude functional redundancy. There have also been several attempts to apply the method to plant and animal parasitic nematodes (e.g. *O. ostertagi* and *H. contortus*) but with variable success (Visser *et al*., 2006; Zawadzki *et al*., 2006; Geldhof *et al*., 2007; Kimber *et al*., 2007; Knox *et al*., 2007; Viney and Thompson, 2008). Viney and Thompson (2008) hypothesised that these failures are due to the inappropriate external supply of dsRNA and/or the functionally defective RNAi pathway in parasitic nematodes. Further RNAi experiments should clarify this hypothesis.

As described in Chapter 4, the *ttl* gene family for species of nematodes of the order Strongylida and *C. elegans* were subdivided in 5 classes based on the conserved amino acids in the first TTL signature domain and the number and location of the cysteine residues. A similar segregation in different classes has been observed in the *C. elegans neuropeptide-like protein* (*nlp*) gene family. Thirty-two *nlp* genes could be subdivided in at least 11 families of putative neuropeptides based on unique motifs (Nathoo *et al*., 2002). Furthermore, the *nlp-4* neuropeptide shows weak similarity to the TTL protein domain while Jacobs *et al*. (2007) suggested a neuronal function for the nematode TTLs. It should be noted that the ineffectiveness of neuronal gene RNAi in *Caenorhabditis elegans* has been reported by several authors (Esposito *et al.*, 2007; Kimber *et al*., 2007). However, in the present study, we were able to knockdown/out 3 out of 5 *C. elegans ttls*, which seems to contradict a neuronal function. Remarkably, Kimber *et al.* (2007) observed that the neuronal FMRFamide-like peptides of the plant nematode *Globodera pallida*, unlike in *C. elegans*, were susceptible to RNAi resulting in aberrant behaviour. More importantly, a recent study by Fleming *et al.* (2007) was able to demonstrate that transthyretin participates in nerve physiology and that it enhances nerve regeneration.

In conclusion, the idea has grown that the members of this diverse TTL protein family may fulfill equally diverse functions which include vitamin A/ retinol and hormone transport, a role in the nervous system and transition to parasitism. Complete knockdown of this gene family in *C. elegans*, additional thyroid, fat and retinol binding assays and analysing the expression profiles of the other Clade V *ttls* are indispensable to gain further insights into their biological role.

4. Conclusions

The host-parasite interface forms a delicate homeostasis between parasite survival strategies and host defence mechanisms. A greater knowledge of the host–parasite relationship on a molecular level would enhance our search for novel drug and vaccine targets. In this thesis we evaluated the cDNA phage display method for the identification of those essential host-parasite interactions. However, our findings have drawn the attention to the fact that a good assessment of the experimental design, in regards to e.g. the complexity of start material, is indispensible for obtaining useful results. Further studies on the phage coat proteins and their assembly in virions hopefully might give some answers upon unsolved problems like display of membrane proteins.

The functional importance of parasite ESPs, involved in regulating internal processes and host-parasite interactions, has been the cornerstone for this thesis. During the functional analysis of the nematode specific genes, we encountered 2 major obstacles. Many novel genes, including the AL gene family, have no homologues in the databases or in *C. elegans*, which makes it very difficult to extrapolate obtained sequence data into useful biological information. Another point of attention is the functional analysis of these nematode specific genes by means of RNAi, e.g. *ttls*. The RNAi technology will assist in unraveling the function of such genes only if there is a recognizable phenotype. However, the combined use of improved genomic, bioinformatic and proteomic tools could provide the solution and should considerably improve our understanding of the complexity of the molecular pathways and responses during parasite infection.

5.References

Ansuini, H., Cicchini, C., Nicosia, A., Tripodi, M., Cortese, R. and Luzzago, A. (2002). Biotin-tagged cDNA expression libraries displayed on lambda phage: a new tool for the selection of natural protein ligands. Nucleic Acids Res 30, e78.

Asojo, O.A., Goud, G., Dhar, K., Loukas, A., Zhan, B., Deumic, V., Liu, S., Borgstahl, G.E. and Hotez, P.J. (2005). X-ray structure of Na-ASP-2, a pathogenesis-related-1 protein from the nematode parasite, *Necator americanus*, and a vaccine antigen for human hookworm infection. J Mol Biol 346, 801-14.

Bair, C., Oppenheim, A., Trostel, A., Prag, G. and Adhya, S. (2008). A phage display system designed to detect and study protein-protein interactions. Mol Microbiol 67, 719-728.

Caillaud, M.C., Dubreuil, G., Quentin, M., Perfus-Barbeoch, L., Lecomte, P., de Almeida Engler, J., Abad, P., Rosso, M.N. and Favery, B. (2008). Root-knot nematodes manipulate plant cell functions during a compatible interaction. J Plant Physiol 165, 104-13.

Castillo, J., Goodson, B. and Winter, J. (2001). T7 displayed peptides as targets for selecting peptide specific scFvs from M13 scFv display libraries. J Immunol Meth 257, 117–122.

Cicchini, C., Ansuini, H., Amicone, L., Alonzi, T., Nicosia, A., Cortese, R., Tripodi, M. and Luzzago, A. (2002). Searching for DNA-protein interactions by lambda phage display. J Mol Biol 322, 697-706.

Clark, J.R. and March, J.B. (2004). Bacterial viruses as human vaccines? Expert Rev Vaccines 3, 463-76.

Cottee, P.A., Nisbet, A.J., ABS El-Osta, Y.G., Webster, T.L. and Gasser, R.B. (2006). Construction of gender-enriched cDNA archives for adult *Oesophagostomum dentatum* by suppressive-subtractive hybridization and a microarray analysis of expressed sequence tags. Parasitol 132, 691-708.

Craig, H., Wastling, J.M. and Knox, D.P. (2006). A preliminary proteomic survey of the in vitro excretory/secretory products of fourth-stage larval and adult *Teladorsagia circumcincta*. Parasitol 132, 535-43.

Cui, L., Rzomp, K.A., Fan, Q., Martin, S.K. and Williams, J (2001). *Plasmodium falciparum*: differential display analysis of gene expression during gametocytogenesis. Exp Parasitol 99, 244-54.

Danner, S. and Belasco, J.G. (2001). T7 phage display: a novel genetic selection system for cloning RNA-binding proteins from cDNA libraries. Proc Nat Acad Sci USA 98, 12954–12959.

Esposito, G., Di Schiavi, E., Bergamasco, C.and Bazzicalupo, P. (2007). Efficient and cell specific knock-down of gene function in targeted *C. elegans* neurons. Gene 395, 170-176.

Fleming, C.E., Saraiva, M.J. and Sousa, M.M. (2007). Transthyretin enhances nerve regeneration. J Neurochem 103, 831-839.

Fortunato, A. and Fraser, A.G. (2005). Uncover genetic interactions in *Caenorhabditis elegans* by RNA interference. Biosci Rep 25, 299-307.

Geldhof, P., Visser, A., Clark, D., Saunders, G., Britton, C., Gilleard, J., Berriman, M. and Knox, D. (2007). RNA interference in parasitic helminths: current situation, potential pitfalls and future prospects. Parasitol 134, 609-19.

Ghosh, A., Srinivasan, P., Abraham, E.G., Fujioka, H. and Jacobs-Lorena, M. (2003). Molecular strategies to study *Plasmodium*-mosquito interactions. Trends Parasitol 19, 94-101.

Gnanasekar, M., Rao, K.V., He, Y.X., Mishra, P.K., Nutman, T.B., Kaliraj, P. and Ramaswamy, K. (2004). Novel phage display-based subtractive screening to identify vaccine candidates of *Brugia malayi*. Infect Immun 72, 4707-15.

Gnanasekar, M., Padmavathi, B. and Ramaswamy, K. (2005). Cloning and characterization of a novel immunogenic protein 3 (NIP3) from *Brugia malayi* by immuno screening of a phage-display cDNA expression library. Parasitol Res 97, 49-58.

Gupta, A., Onda, M., Pastan, I., Adhya, S. and Chaudhary, V.K. (2003). High-density functional display of proteins on bacteriophage lambda. J Mol Biol 334, 241-54.

Hawdon, J. M., Jonest, B. F., Hoffman, D. R. and Hotez, P. J. (1996). Cloning and characterization of *Ancylostoma*-secreted protein. A novel protein associated with the transition to parasitism by infective hookworm larvae. J Biol Chem 271, 6672-6678.

Hawdon, J. M., Narasimhan, S. and Hotez, P. J. (1999). Ancylostoma secreted protein 2: cloning and characterization of a second member of a family of nematode secreted proteins from *Ancylostoma caninum*. Mol Biochem Parasitol 30, 149-165.

Hoess, R.H. (2002). Bacteriophage lambda as a vehicle for peptide and protein display. Curr Pharm Biotechnol 3, 23-8.

Hufton, S.E., Moerkerk, P.T., Meulemans, E.V., de Bruïne, A., Arends, J.W. and Hoogenboom, H.R. (1999). Phage display of cDNA repertoires: the pVI display system and its applications for the selection of immunogenic ligands. J Immunol Methods 231, 39-51.

Jacob, J., Vanholme, B., Haegeman, A. and Gheysen, G. (2007). Four transthyretin-like genes of the migratory plant-parasitic nematode *Radopholus similis*: members of an extensive nematode-specific family. Gene 402, 9-19.

Jefferies, D. (1998). Selection of Novel Ligands from Phage Display Libraries: An Alternative Approach to Drug and Vaccine Discovery? Parasitol Today 14, 202-206.

Jespers, L.S., Messens, J.H., De Keyser, A., Eeckhout, D., Van Den Brande, I.,Gansemans, Y.G., Lauwereys, M.J., Vlasuk, G.P. and Stanssens, P.E. (1995). Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI. Biotechn (N.Y.) 13, 378-382.

Kimber, M.J., McKinney, S., McMaster, S., Day, T.A., Fleming, C.C. and Maule, A.G. (2007). *flp* gene disruption in a parasitic nematode reveals motor dysfunction and unusual neuronal sensitivity to RNA interference. FASEB J 21, 1233-43.

Knox, D.P., Geldhof, P., Visser, A. and Britton, C. (2007). RNA interference in parasitic nematodes of animals: a reality check? Trends Parasitol 23, 105-7.

Lanzillotti, R. and Coetzer, T.L. (2008). Phage display: a useful tool for malaria research? Trends Parasitol 24, 18-23.

Lauterbach, S.B., Lanzillotti, R. and Coetzer, T.L. (2003). Construction and use of *Plasmodium falciparum* phage display libraries to identify host parasite interactions. Malar J 2, 47.

Li, B.W., Rush, A.C., Crosby, S.D., Warren, W.C., Williams, S.A., Mitreva, M. and Weil, G.J. (2005). Profiling of gender-regulated gene transcripts in the filarial nematode *Brugia malayi* by cDNA oligonucleotide array analysis. Mol. Biochem. Parasitol. 143, 49-57

Lizotte-Waniewski, M., Tawe, W., Guiliano, D.B., Lu, W., Liu, J., Williams, S.A. and Lustigman, S. (2000). Identification of potential vaccine and drug target candidates by expressed sequence tag analysis and immunoscreening of *Onchocerca volvulus* larval cDNA libraries. Infect Immun 68, 3491-501.

Mizuki, N. and Kasahara, M. (1992). Mouse submandibular glands express an androgen-regulated transcript encoding an acidic epididymal glycoprotein-like molecule. Mol Cell Endocrinol 89, 25-32.

Moser, J.M., Freitas, T., Arasu, P. and Gibson, G. (2005). Gene expression profiles associated with the transition to parasitism in *Ancylostoma caninum* larvae. Mol Biochem Parasitol 143, 39-48.

Murray, J., Gregory, W. F., Gomez-Escobar, N., Atmadja, A. K. and Maizels, R. M. (2001). Expression and immune recognition of *Brugia malayi* VAL-1, a homologue of vespid venom allergens and *Ancylostoma* secreted proteins. Mol Biochem Parasitol 118, 89-96.

Nathoo, A.N., Moeller, R.A., Westlund, B.A. and Hart, A.C. (2002). Identification of neuropeptide-like protein gene families in *Caenorhabditis elegans* and other species. Proc Natl Acad Sci U S A 98, 14000-5.

Nisbet, A.J. and Gasser, R.B. (2004). Profiling of gender-specific gene expression for *Trichostrongylus vitrinus* (Nematoda: Strongylida) by microarray analysis of expressed

sequence tag libraries constructed by suppressive-subtractive hybridization. Int. J Parasitol. 34, 633–643

Nisbet, A.J., Redmond, D.L., Matthews, J.B., Watkins, C., Yaga, R., Jones, J.T., Nath, M. and Knox, D.P. (2008). Stage-specific gene expression in *Teladorsagia circumcincta* (Nematoda: Strongylida) infective larvae and early parasitic stages. Int J Parasitol, in press.

Parkinson, J., Mitreva, M., Whitton, C., Thomson, M., Daub, J., Martin, J., Schmid, R., Hall, N., Barrell, B., Waterston, R.H., McCarter, J.P. and Blaxter, M.L. (2004). A transcriptomic analysis of the phylum *Nematoda*. Nat Genet 36,1259-67.

Reinke, V., Smith, H.E., Nance, J., Wang, J., Van Doren, C., Begley, R., Jones, S.J., Davis, E.B., Scherer, S., Ward, S. and Kim, S.K. (2000). A global profile of germline gene expression in *C. elegans*. Mol Cell 6, 605-16.

Reinke, V., Gil, I.S., Ward, S. and Kazmer, K. (2004). Genome-wide germline-enriched and sex-biased expression profiles in *Caenorhabditis elegans*. Development 131, 311-323

Robinson, M.W., Greig, R., Beattie, K.A., Lamont, D.J. and Connolly, B. (2007). Comparative analysis of the excretory-secretory proteome of the muscle larva of *Trichinella pseudospiralis* and *Trichinella spiralis*. Int J Parasitol 37, 139-48.

Tavernier, J., Eyckerman, S., Lemmens, I., Van der Heyden, J., Vandekerckhove, J. and Van Ostade, X. (2002). MAPPIT: a cytokine receptor-based two-hybrid method in mammalian cells. Clin Exp Allergy 32, 1397-404.

Viaene, A., Crab, A., Meiring, M., Pritchard, D. and Deckmyn, H. (2001). Identification of a collagen-binding protein from *Necator americanus* by using a cDNA-expression phage display library. J Parasitol 87, 619-625.

Viney, M.E. and Thompson, F.J. (2008). Two hypotheses to explain why RNA interference does not work in animal parasitic nematodes. Int J Parasitol 38, 43-47.

Visser, A., Geldhof, P., de Maere, V., Knox, D.P., Vercruysse, J. and Claerebout, E. (2006). Efficacy and specificity of RNA interference in larval life-stages of *Ostertagia ostertagi*. Parasitol 133, 777-83.

Visser, A., Van Zeveren, A.M., Meyvis, Y., Peelaers, I., Van den Broeck, W., Gevaert, K., Vercruysse, J., Claerebout, E., Geldhof, P. (2008). Gender-enriched transcription of activation associated secreted proteins in *Ostertagia ostertagi*. Int J Parasitol 38, 455-65.

Willats, W.G. (2002). Phage display: practicalities and prospects. Plant Mol Biol 50, 837-54.

Yang, J., Wu, W. and Zhu, J. (2008). Mapping interspecific genetic architecture in host-parasite interaction system. Genetics, in press.

Yao, L., Yin, J., Zhang, X., Liu, Q., Li, J., Chen, L., Zhao, Y., Gong, P. and Liu, C. (2007). *Cryptosporidium parvum*: identification of a new surface adhesion protein on sporozoite and oocyst by screening of a phage-display cDNA library. Exp Parasitol 115, 333-8.

Yatsuda, A.P., Krijgsveld, J., Cornelissen, A.W., Heck, A.J. and de Vries, E. (2003). Comprehensive analysis of the secreted proteins of the parasite *Haemonchus contortus* reveals extensive sequence variation and differential immune recognition. J Biol Chem 278, 16941-51.

Zhang, Q., Bai, G., Cheng, J., Yu, Y., Tian, W. and Yang, W. (2007). Use of an enhanced green fluorescence protein linked to a single chain fragment variable antibody to localize *Bursaphelenchus xylophilus* cellulase. Biosci Biotechnol Biochem 71, 1514-20.

Zawadzki, J.L., Presidente, P.J., Meeusen, E.N. and De Veer, M.J. (2006). RNAi in *Haemonchus contortus:* a potential method for target validation. Trends Parasitol 22, 495-9.

Zucconi, A., Dente, L., Santonico, E., Castagnoli, L. and Cesareni, G. (2001). Selection of ligands by panning of domain libraries displayed on phage lambda reveals new potential partners of synaptojanin 1. J Mol Biol 307, 1329–1339.

Summary

Infections with gastrointestinal nematodes influence livestock worldwide and result in parasitic gastroenteritis (PGE). This disease places a substantial economic burden on the cattle industry due to production losses. In temperate climate regions, the most important and pathogenic of these gastrointestinal nematodes is *Ostertagia ostertagi*, which infects the abomasum. Control of PGE in Europe is mainly based on the use of anthelmintics. However, the increasing incidence of anthelmintic resistance poses a serious threat on the future routine use of anthelmintics and has stimulated the search for alternative control measures. Vaccination is one of the most feasible alternatives (**General introduction**).

During its development, *O. ostertagi* induces several morphological, physiological and biochemical changes in the abomasum of the host. These changes (described in detail in the **first part of chapter 1**) result in tissue damage, a substantial increase in the pH of the abomasal contents, loss of functional activity of parietal cells, elevated plasma pepsinogen levels, hypoalbuminaemia and hypergastrinaemia. Also, *O. ostertagi* induces a strong Th2 type immune response, accompanied by accumulation of local antibodies, mast cells and eosinophils. Although the mechanisms or processes used by the parasite to change the abomasal environment are largely unknown, parasite excretory-secretory products (ESPs) are considered to play an important role. ESPs comprise all of the material released by the parasites *in vitro* and presumably also *in vivo*. ESPs are often stage-specific and may be derived from the parasite surface, from specialized secretory glands or as by-products of parasite digestion. They are thought to play an important role in host penetration, parasite feeding and reproduction but also take part in parasite-mediated immunosuppression. Numerous *in vitro* and *in vivo* studies have assessed the role of parasite ESPs (such as *Teladorsagia circumcincta* and *Haemonchus contortus*) in the pathogenesis of PGE. However, data on the involvement of *O. ostertagi* ESPs in the pathogenesis are scarce and mainly focussed on their immunomodulatory properties. This is described in the **second part of chapter 1.** Because specific parasite ESPs involved in host-parasite interactions have not been defined yet, we have reviewed known *O. ostertagi* ESPs that might have a crucial role in the host-parasite homeostasis.

The overall objective of this thesis was to study *Ostertagia ostertagi* ESPs and their possible interaction with bovine abomasal proteins. Identification of interacting ESPs could open new perspectives for vaccination research and drug development.

In **chapter 2** we have assessed the use of the filamentous M13 pVI phage display system to identify the protein-protein interactions involved in the pathophysiological and immunological alterations in the bovine abomasum during an infection with *Ostertagia*. The phage display technology allows functional

display of foreign proteins on the surface of the bacteriophage by means of translational fusion to the C-terminus of the minor phage coat protein pVI. Native ESPs from the L4 and adult life stage of the parasite were screened against a phage display cDNA library produced from abomasal cells of infected calves. However, the identified abomasal proteins were not involved in the pathophysiological changes during an *Ostertagia* infection or were localized intracellularly. It is therefore unlikely that these interactions with *Ostertagia* ESPs are relevant under *in vivo* conditions. A second experiment was set up, in which native membrane proteins of the abomasal epithelial cells were screened against an adult *O. ostertagi* phage display library. In this set-up, each panning round seems to have enriched for phages, displaying a very short random peptide, with no homology to any known nematode protein. Although the phage display technology has shown great potential to study protein-protein interactions, we were unable to identify specific interactions between ESPs and the abomasal proteins with phage display.

However, the importance of ESPs during parasite infection remains unquestionable. Furthermore, increasing incidence of anthelmintic resistance in parasitic nematodes has resulted in the extensive search for appropriate vaccine and drug candidates, which ideally are nematode-specific, conserved and essential for the parasite's survival and/or development. Analysis of the expressed sequence tag (EST) sequences from 30 species across the phylum Nematoda, identified hundreds of these nematode-specific and conserved gene families. Two such nematode-specific and secreted protein families were characterised in the remainder of this project.

An adult *O. ostertagi* ESP fraction, obtained by thiol-sepharose chromatography (ES-thiol), has previously shown to partially protect cattle against challenge infection in two independent vaccination experiments. Mass spectrometric (MS) analysis indicated that the most abundant antigens present in this fraction were two activation-associated secreted proteins, termed Oo-ASP1 and Oo-ASP2. A recent reanalysis of these MS data by comparison against the currently available *O. ostertagi* expressed sequence tag (EST) dataset identified additional ESPs that were described elaborately in **chapter 3**. They belong to the nematode-specific ASP protein family, which is characterised by the SCP/Tpx-1/Ag5/PR-1/Sc7 motif. Sequence analyses demonstrated that although these novel ESPs show homology to other ASPs, they are clearly different. Therefore, the proteins were named Oo-AL1 and Oo-AL2 (*O. ostertagi* ASP-like protein). Analysis of the nematode genome and EST databases indicated that these novel ES proteins are unique for *O. ostertagi* and the closely related *T. circumcincta.* The AL proteins of these parasites (Oo-AL1, OoAL2, Tc-AL1, Tc-Al2) contain a signal peptide and one predicted N-glycosylation site. By means of RT-PCR, the highest level of transcription of *Oo-al1* and *Tc-al1* were detected in the adult and L4 life stage, respectively. The transcripts of the shorter variants *Oo-al2* and *Tc-al2* were hardly detectable. Western blots of somatic extracts and ES products of

different developmental stages of *O. ostertagi,* developed with anti-Oo-AL1 antibodies, inferred the presence of Oo-AL proteins in the ES products of adult worms. The Western blot of the non-reduced adult ESPs revealed a higher band, suggesting dimerisation of the OoAL proteins under native conditions. PCR was performed on genomic DNA from pooled and individual *O. ostertagi* adults to isolate Oo-al encoding genes. Three predicted *Oo-al* genes with a similar structure of 7 exons and 6 introns were isolated. The function of the nematode ASPs and AL proteins is still unknown. The SCP/TPX-1/Ag5/PR-1/Sc7 protein domain is found in a broad range of proteins and their suggested functions are equally diverse. It has been postulated that ASPs take part in the infection process and the transition to parasitism. More importantly, no homologous AL-like proteins were identified in any other nematode species, both parasitic and free-living. This information suggests a key functional role for the AL proteins in the life cycle of these abomasal parasites. Furthermore, the AL sequences from *O. ostertagi* and *T. circumcincta* show notable sequence diversity between the species and a slightly different transcription pattern, which could relate to an adaptation to cattle and small ruminants, respectively.

In **chapter 4**, we investigated another nematode-specific, secreted protein family, namely the transthyretin like protein (TTL) family in *O. ostertagi.* The corresponding TTL gene family is one of the largest conserved nematode-specific gene families, coding for a group of proteins with sequence similarity to transthyretins (TTR) and transthyretin-related proteins (TRP). EST database mining revealed the presence of at least 18 *Oo-ttl* genes in *O. ostertagi.* Evaluation of their stage- and gender-specific transcription patterns by RT-PCR, demonstrated that most of them are constitutively transcribed from the non-parasitic L3 stage onwards. The full-length cDNA of one of these genes (*Oo-ttl-1*) was obtained by a PCR approach and cloned for recombinant expression. The predicted protein sequence Oo-TTL-1 contained a signal peptide and was characterised by a 'TTL' protein domain, which consists of two conserved signature motifs. Western blotting with specific antiserum showed that the native Oo-TTL-1 was mainly present in the ESPs of adult parasites. The protein was immunolocalized to the pseudocoelomic fluid of adult worms. A phylogenetic analysis of all TTL proteins of Clade V nematoda showed that they could be divided into at least 7 different classes of TTLs. This classification is based on conserved amino acids in the first TTL signature domain and the number and location of the cysteine residues. The biological role of the TTLs in nematode biology is still unclear. Based on their relationship with TTRs and TRPs, a number of functions have been postulated: carriers of hormones, digestion/ absorption of nutrients and a putative role in the nervous system. A theoretical 3D model of Oo-TTL-1 indicated that the protein may have a similar structure to TTRs, i.e. beta-sheets which are arranged in a beta-sandwich. However, the particular beta-strand involved in dimerisation or tetramerisation is not present and this is in agreement with the results of the Western blotting which showed that under

non-denaturing conditions, Oo-TTL-1 still migrates at 15 kDa. Furthermore, in contrast to TTRs, competitive binding studies with recombinant Oo-TTL-1 indicated that the protein was devoid of any hydrophobic ligand- or thyroid hormone-binding properties. Finally, combinatorial RNAi on five *ttl* genes in the free-living nematode *Caenorhabditis elegans* did not induce any visible phenotype. The lack of a phenotype might be due to the functional redundancy within this large gene family. To investigate this, we should consider combinatorial RNAi on a whole class of *ttl* genes. In addition, more information on the transcription profile and tissue distribution of the TTLs in *C. elegans* should be collected in order to provide new insights in the biological role of the TTLs in nematodes.

In **chapter 5**, we provided the reader with some new insights on tools to study host-parasite interactions. One could proclaim: Why use phage display? Over the last decades, numerous reports obtained by phage display have yielded insight into parasitic diseases, especially for probing molecular interactions between the parasite and the host. Undoubtedly, this technology is extremely powerful, versatile, cheap, rapid to set up and requires no special equipment. In this thesis, we utilized the filamentous M13 pVI cDNA phage display method to overcome the limitations of direct fusions of cDNA to the N-terminus of phage coat proteins pIII or pVIII due to transcriptional stop codons present in the 3' end of the cDNA. However, a pivotal drawback of using filamentous phage display remains the non-lytic propagation mechanism of this phage that requires export of all the phage coat components through the bacterial inner membrane before phage assembly. In principle, this limitation may be avoided by using the lytic phages Lambda and T7, in which capsid assembly occurs entirely in the cytoplasm prior to cell lysis. Recently, a T7 phage display screenings method was developed based on *Cryptosporidium parvum* cDNA in order to identify a new surface adhesion protein on sporozoite and oocyst. Furthermore, recent studies have shown that unlike T7, Lambda phage can tolerate the display of relative large proteins at high density. A novel lambda gpD vector was designed to highly enrich for clones containing open reading frames (ORFs). *In vivo* biotinylation of the fusion phage during amplification followed by streptavidin affinity chromatography resulted in a higher percentage of clones with the correct ORF and a low background of false positive clones. Additionally, different phage types such as T7 and M13 may be combined in one experiment.

Each of these phage display (M13, T7, Lambda) methods has its own advantages and disadvantages with respect to each particular application. It should be noted that phage display is just one of many tools employed in parasitic research. For example, alternative methods include the MAPPIT technique (the Mammalian Protein-Protein Interaction Trap), proteomics and genomic tools such as the differential display technique, subtractive cDNA hybridisation and the microarray technology. They could aid in the identification of key parasite and host proteins during infection.

Furthermore, in chapter 5, we further discussed the putative function of the two nematode specific protein families (the ASP like and Transthyretin like protein families) that were analysed in this PhD thesis. First, it has been postulated that ASPs and the AL proteins may be involved in the infection process and the transition to parasitism. However, a recent study revealed a male enriched transcription pattern in several *O. ostertagi* ASP genes, indicating a role in reproduction or in the development of the reproductive system. This confers with their similarity to the SCP/Tpx-1/Ag5/PR-1/Sc7 protein family, which is comprised of sperm coating proteins, acidic epididymal glycoproteins, testis specific proteins and cysteine rich secreted proteins. Alternatively, the male enrichment on transcriptional level of many of the ASPs in *O. ostertagi* might be a remnant of an ancestral ASP molecule that might once have had an important function in male reproduction. Unfortunately, RNAi in *C. elegans* cannot be used to unravel the functional role of the AL proteins because *C. elegans* does not have any homologues that can be targeted. Secondly, we discussed the TTL protein family in Clade V nematodes. It has been postulated that the members of this diverse protein family may fulfill equally diverse functions, which include transition to parasitism and a role in the nervous system. Interestingly, a similar segregation in different classes has been observed in the *C. elegans neuropeptide-like protein* (*nlp*) gene family. Furthermore, recent studies demonstrated that TTR participates in nerve physiology and that neuronal peptides of a plant nematode, unlike in *C. elegans*, were susceptible to RNAi resulting in aberrant behaviour. Therefore, complete knockdown of this gene family in *C. elegans*, additional thyroid, fat and retinol binding assays and analysing the expression profiles of the other Clade V *ttls* are indispensable to gain further insights into their biological role.

In conclusion, the combined use of improved genomics, bioinformatics and proteomics should considerably improve our understanding of the complexity of the molecular pathways and responses during parasite infection. Hence, a greater knowledge of the host–parasite relationship should increase the number of cellular functions identified, which can be targeted by drugs and could result in the identification of new vaccine antigens.

Samenvatting

Besmettingen met gastrointestinale (GI) nematoden veroorzaken wereldwijd parasitaire gastroenteritis (PGE) bij rundvee. Deze ziekte is verantwoordelijk voor grote economische verliezen in de rundveehouderij die toe te schrijven zijn aan o.a. productieverliezen. De meest voorkomende en pathogene van deze GI nematoden is *Ostertagia ostertagi*, een belangrijke lebmaagnematode bij runderen in gematigde klimaatgebieden. In Europa is de controle van PGE hoofdzakelijk gebaseerd op het gebruik van ontwormingsproducten of anthelminthica. De toenemende resistentie tegen de beschikbare anthelminthica heeft de zoektocht naar alternatieve controlestrategieën gestimuleerd. Vaccinatie wordt als één van de beste alternatieven beschouwd (**Algemene inleiding)**.

Tijdens zijn ontwikkeling veroorzaakt *O. ostertagi* belangrijke morfologische, pathofysiologische en biochemische veranderingen in het lebmaagmilieu van zijn gastheer. Deze veranderingen zijn gedetailleerd beschreven in **het eerste deel van hoofdstuk 1** en resulteren in epitheelschade, verhoogde pH van de lebmaaginhoud, verminderde activiteit van de parietaalcellen, verhoogde bloed-pepsinogeen niveaus, hypoalbuminemie en hypergastrinemie. Bovendien induceert *O. ostertagi* ook een sterke T helper 2 respons die gekenmerkt wordt door accumulatie van lokale antilichamen, mastcellen en eosinofielen. Over de onderliggende moleculaire mechanismen en specifieke eiwit-eiwit interacties betrokken in het infectieproces ontbreekt echter nog alle informatie. Een belangrijke rol in deze processen is toegeschreven aan parasiet excretie-secretie producten (ESPs). ESPs zijn moleculen die dikwijls stadiumspecifiek door de nematoden uitgescheiden worden *in vitro* en vermoedelijk ook *in vivo.* ESPs worden geëxpresseerd aan het oppervlak van de parasiet, vrijgesteld door gespecialiseerde excretie/secretie organen of uitgescheiden als bijproducten van de digestie. Ze worden niet alleen belangrijk geacht bij kritieke biologische functies zoals het binnendringen van de gastheer, de voeding en de voortplanting van de parasiet, maar ook bij het ontwijken van anti-parasitaire immuunresponsen. Verschillende *in vitro* en *in vivo* studies hebben de rol van parasiet ESPs (o.a. bij *Teladorsagia circumcincta* en *Haemonchus contortus*) in de pathogenese van PGE bestudeerd. Informatie over de invloed van *O. ostertagi* ESPs in de pathogenese is echter schaars en vooral gefocust op hun immunomodulatorische eigenschappen. Dit werd beschreven in het **tweede deel van hoofdstuk 1**. Tot nu toe werden nog geen specifieke parasiet ESPs geïdentificeerd die betrokken zijn in de gastheer-parasiet interactie. Daarom werd een overzicht gegeven van gekende *O. ostertagi* ESPs die mogelijks een cruciale rol hebben in de gastheer-parasiet homeostase.

Het uitgangspunt van dit onderzoek was het beter begrijpen van de samenstelling van de parasiet ESPs en hun mogelijke interactie met runderlebmaag eiwitten. Dit kan nieuwe perspectieven openen voor de identificatie van wormantigenen met een chemotherapeutisch en protectief potentieel.

In **hoofdstuk 2** werd het gebruik van het filamenteuze M13 pVI faag display systeem geëvalueerd om de *O. ostertagi* (ESPs) eiwit-lebmaageiwit interacties te identificeren. De faag display technologie is gebaseerd op het feit dat fusies van vreemde sequenties met een 'minor phage coat' proteïne (vb. pVI) gemaakt kunnen worden zodanig dat het vreemd eiwit in een actieve vorm aan de buitenkant van de faag bereikbaar is. Natieve ESPs van L4 larven en adulte wormen werden gescreend tegen een faag display bank van abomasale cellen. De geïdentificeerde lebmaagproteïnen waren echter niet betrokken bij de verandering van abomasale pH, immunomodulatie, celhyperplasie, enz. of zijn intracellulair gelokaliseerd. Het is daarom onwaarschijnlijk dat deze interacties met *O. ostertagi* ES materiaal *in vivo* relevant zijn. Een tweede experiment werd uitgevoerd, waarin membraanproteïnen van de abomasale epitheliale cellen gescreened werden tegen een *O. ostertagi* faag display bank. In deze opstelling bleek elke panningronde echter bacteriofagen aan te rijken die alleen zeer korte willekeurige peptiden expresseren. Bovendien vertoonden deze peptiden geen homologie met reeds gekende wormproteïnes. Hoewel de faag display technologie potentieel heeft getoond om eiwit-eiwit interacties te bestuderen, wordt nu duidelijk dat deze techniek beperkingen heeft wanneer ze gebruikt wordt om screenings uit te voeren met volledige cDNA banken.

Niettegenstaande er met de faag display techniek geen specifieke interacties tussen *Ostertagia* ESPs en eiwitten uit de runderlebmaag konden aangetoond worden, staat het belang van ESPs in het infectieproces buiten kijf. Bovendien heeft de dreigende anthelminthicum-resistentie geresulteerd in de zoektocht naar geschikte vaccinkandidaten en doelwitten voor geneesmiddelen. Idealiter zijn deze kandidaten nematoden-specifiek, geconserveerd en essentieel voor de overleving en/of ontwikkeling van de parasiet. Door de analyse van de 'expressed sequence tag '(EST) sequenties van 30 species van het phylum Nematoda, werden honderden nematoden-specifieke en geconserveerde genfamilies geïdentificeerd. Daarom werden in het vervolg van dit project twee nematoden-specifieke en gesecreteerde eiwitfamilies gekarakteriseerd.

Tijdens 2 onafhankelijke vaccinatie-experimenten werd aangetoond dat immunisatie met een adulte *O. ostertagi* ESP fractie (ES-thiol), opgezuiverd door middel van thiol-sepharose chromatografie, in staat was om runderen gedeeltelijk te beschermen tegen herinfectie. Massaspectrometrie (MS) toonde aan dat 2 'activation-associated secreted' proteïnes (Oo-ASP1 en 2) de meest abundante antigenen waren in deze protectieve fractie. Recent werden deze MS gegevens opnieuw geanalyseerd door te 'blasten' tegen een geactualiseerde *O. ostertagi* EST databank. Hierbij werden additionele ESPs geïdentificeerd die uitvoerig besproken werden in **hoofdstuk 3**. Ze behoren tot een nieuwe, nematoden-specifieke eiwitfamilie met het SCP/Tpx-1/Ag5/PR-1/Sc7 motief. Hoewel dit eiwitmotief ook aanwezig is in de ASP eiwitfamilie van nematoden, toonden sequentie analyses aan dat de nieuwe ES proteïnen verschillen van de eerder geïdentificeerde ASPs. Ze werden daarom Oo-AL1 en Oo-Al2 (*O. ostertagi*

ASP-like proteïne) genoemd. Analyses van het nematodengenoom en EST databanken toonden aan dat deze nieuwe AL eiwitten uniek zijn voor *O. ostertagi* en de nauw verwante *T. circumcincta*. De AL proteïnes van deze parasieten (Oo-AL1, OoAL2, Tc-AL1, Tc-Al2) bevatten een signaalpeptide en één voorspelde N-glycosylatie site. Door middel van RT-PCR werden de hoogste transcriptieniveaus van *Oo-al1* en *Tc-al1* gedetecteerd in respectievelijk het adulte en L4 stadium. De kortere varianten *Oo-al2* en *Tc-al2* vertoonden bijna geen transcriptie. Western Blots van somatische extracten en ES producten van *O. ostertagi* L4s en adulten met anti-Oo-AL1 antilichamen bevestigden het stadium-specifieke expressiepatroon van de AL proteïnen in adulte ES producten. Een Western blot van niet gedenatureerd adult ES materiaal toonde een hogere band die op dimerisatie van het Oo-AL eiwit kan wijzen. PCRs werden uitgevoerd op genomisch DNA van gepoolde en individuele *O. ostertagi* wormen om de *Oo-al* coderende genen te detecteren. Drie genen met een gelijkaardige structuur van 7 exons en 6 introns werden geïsoleerd. De functie van de nematode ASPs en AL proteïnen is nog ongekend. Het SCP/TPX-1/Ag5/PR-1/Sc7 proteïne-domein werd geïdentificeerd in een brede waaier van proteïnen met even diverse functies. Voor ASPs werd een rol gesuggereerd tijdens het infectieproces en de overgang naar parasitisme. Er zijn geen homologe AL eiwitten geïdentificeerd in andere nematoden, zowel vrijlevend als parasitair. Dit wijst op een zeer specifieke functie voor de AL proteïnen in de levenscyclus van abomasale, niet-bloed voedende parasieten. De AL sequenties van *O. ostertagi* en *T. circumcincta* vertonen bovendien opmerkelijke onderlinge verschillen en hebben een licht verschillend transcriptiepatroon. Dit zou op een gastheerspecifieke adaptatie kunnen wijzen.

In **hoofdstuk 4** werd een andere nematoden-specifieke en gesecreteerde eiwitfamilie met therapeutisch potentieel bestudeerd, namelijk de transthyretin-like (TTL) eiwitfamilie (PF01060). De corresponderende TTL genfamilie is één van de grootste, conserveerde en nematoden-specifieke gen families die codeert voor een groep van eiwitten met enige gelijkenis aan de transthyretines (TTR) en transthyretin-related proteïnen (TRP). Door middel van EST database screenings werden ten minste 18 *Oo-ttl* genen geïdentificeerd die merendeels constitutief overgeschreven werden vanaf het niet-parasitair L3 stadium. De volledige cDNA sequentie van één van deze genen (*Oo-ttl-1*) werd verkregen door middel van PCR en gekloneerd voor recombinante expressie. Het Oo-TTL-1 eiwit bevatte een signaalpeptide en werd gekenmerkt door een 'TTL' proteïne domein, dat opgebouwd is uit 2 geconserveerd motieven. Western blotting met specifiek antiserum toonde een sterk signaal in adult ES materiaal. Vervolgens werd het Oo-TTL1 eiwit geimmunolocaliseerd in de interstitiële ruimte van adulte mannetjes en vrouwtjes. Door middel van een fylogenetische analyse konden alle TTL proteïnen van de Klasse V nematoden onderverdeeld worden in ten minste 7 verschillende groepen. Deze classificatie werd gebaseerd op de geconserveerde aminozuren van het eerste TTL motief en op het aantal en de lokalisatie van

cysteïne residu's. De biologische relevantie van TTLs in de nematodenbiologie is nog onduidelijk. Op basis van hun verwantschap met de TTRs en TRPs zijn er verschillende hypotheses: dragers van hormonen, vertering of absorptie van nutriënten en een rol in het zenuwstelsel. Het theoretisch 3D model van Oo-TTL-1 vertoonde een gelijkaardige opbouw als de TTRs, namelijk betabladen gerangschikt in een beta-sandwich. De specifieke betastreng betrokken in dimerisatie of tetramerisatie was echter afwezig en dit stemt overeen met de resultaten van de Western blot onder niet denaturerende condities. Bovendien toonden competitieve bindingstudies aan dat in tegenstelling tot TTR, recombinant Oo-TTL-1 geen affiniteit heeft voor hydrofobe liganden of thyroid hormonen. Finaal werd er een gecombineerd RNAi experiment uitgevoerd op 5 *ttl* genen in de vrij-levende nematode *Caenorhabditis elegans*, maar er kon geen afwijkend fenotype waargenomen worden. Dit gebrek aan fenotype kan te wijten zijn aan de functionele redundantie binnen deze grote genfamilie. Gecombineerde RNAi op de totale klasse van *ttl* genen zou een oplossing kunnen zijn voor dit probleem. Tot besluit, meer informatie over de transcriptieprofielen en weefseldistributie van de TTLs in *C. elegans* is noodzakelijk om een beter inzicht te krijgen in de biologische rol van TTLs in nematoden.

In **hoofdstuk 5** hebben we enkele nieuwe hulpmiddelen doorgelicht die aangewend kunnen worden voor het bestuderen van de gastheer-parasiet interactie. Een voor de hand liggende vraag is 'Waarom kiest men voor de faag display technologie?' Gedurende de laatste decennia hebben talrijke faag display experimenten bijgedragen tot een beter inzicht in parasitaire ziekten, meerbepaald door het analyseren van de moleculaire interactie tussen de parasiet en de gastheer. Deze technologie is ongetwijfeld krachtig, veelzijdig, goedkoop, snel op te starten en er is bovendien geen speciale apparatuur vereist. Aangezien transcriptionele stopcodons aan het 3' eind van het cDNA directe fusies van cDNA aan de N-terminus van de kapseleiwitten pIII of pVIII verhinderen, hebben we in deze thesis gebruik gemaakt van de filamenteuze M13 pVI cDNA faag display. Nochtans blijft de niet-lytische vermeerderingscyclus van filamenteuze bacteriofagen de belangrijkste "bottleneck" omdat alle faagmantel componenten vóór faag assemblage geëxporteerd moeten worden door het bacteriële binnenmembraan. Het gebruik van lytische bacteriofagen Lambda en T7 verhelpt dit probleem aangezien de assemblage van de faagmantel volledig in het cytoplasma gebeurt vooraleer cellysis optreedt. Recent werd door middel van T7 faag display met *Cryptosporidium parvum* cDNA een nieuw oppervlakte adhesie proteïne geïdentificeerd op sporozoite en oocyst. Bovendien hebben recente studies aangetoond dat, in tegenstelling tot T7, de bacteriofaag Lambda de vertoning van relatieve grote proteïnen bij een hoge densiteit kan tolereren. Daarnaast werd een nieuwe lambda gpD vector ontworpen om klonen aan te verrijken die een correct open leesraam(ORF) bevatten. *In vivo* biotinylatie van de fusiefaag, gevolgd door een streptavidine affiniteitschromatografie, resulteerde in een hoger percentage klonen met

correcte ORF en een lagere achtergrond van vals positieve klonen. Bovendien kunnen de verschillende phage types zoals T7 en M13 in één experiment worden gecombineerd.

Elk van deze faag display (M13, T7, Lambda) methodes wordt gekenmerkt door zijn eigen voor- en nadelen met betrekking tot elke specifieke toepassing. Bovendien is faag display slechts één van vele hulpmiddelen die in parasitair onderzoek worden aangewend om de gastheer-parasiet homeostase te bestuderen. Alternatieve methodes zijn o.a. de MAPPIT techniek (de zoogdier proteïne-proteïne interactie trap), proteomics en 'genomic' methodes zoals de differentiële display, subtractieve DNA hybridisatie en de micro-array technologie. Deze technieken kunnen een belangrijke bijdrage leveren tot de identificatie van parasiet en gastheerproteïnen, betrokken in het infectieproces.

Voorts werd in hoofdstuk 5 de hypothetische functie van de twee nematoden-specifieke eiwitfamilies (ASP-like en Transthyretin-like eiwitfamilies) verder geanalyseerd. Aanvankelijk werd gestipuleerd dat ASPs en de AL proteïnen betrokken zijn in het infectieproces en de overgang naar parasitisme. Een recente studie toonde echter een mannelijk verrijkt transcriptiepatroon aan in verschillende *O. ostertagia* ASP genen, wat een rol in reproductie of in de ontwikkeling van het reproductieve systeem zou kunnen suggereren. Nochtans zou dit eerder op een restant van een voorouderlijke ASP molecule kunnen wijzen met een belangrijke functie in mannelijke reproductie. Aangezien *C. elegans* geen AL homologen bezit, kan de RNAi techniek in *C. elegans* niet gebruikt worden om de functie van de AL eiwitten te ontrafelen. Vervolgens werd de TTL eiwitfamilie in Clade V besproken. Recent werd gesuggereerd dat proteïnen behorend tot deze diverse TTL familie even diverse functies kunnen vervullen zoals bijvoorbeeld een rol in de overgang naar parasitisme en een rol in het zenuwstelsel. Eerder werd een vergelijkbare segregatie in verschillende klassen waargenomen in de 'neuropeptide-like' (nlp) genfamilie van *C. elegans*. Bovendien demonstreerden recente studies dat TTR deelneemt aan de zenuwfysiologie en dat de plantnematode neuronenpeptiden wel vatbaar waren voor RNAi in tegenstelling tot in *C. elegans*, wat resulteerde in afwijkend gedrag. Toekomstig onderzoek naar hun biologische rol zal zich dus moeten richten op complete uitschakeling van deze *ttl* genfamilie in *C. elegans*, additionele thyroid-, vet- en retinol-bindingsstudies en transcriptieprofiel-analyses van andere Clade V ttls.

Tot besluit, de combinatie van verbeterde genomics, bioinformatica en proteomics zou in belangrijke mate bijdragen tot het beter begrijpen van de onderliggende moleculaire mechanismen gedurende een parasietinfectie. Een grotere kennis van het 'partnership' tussen gastheereiwitten en parasitaire liganden zou bovendien nieuwe perspectieven openen voor de identificatie van wormantigenen met een chemotherapeutisch en protectief potentieel.